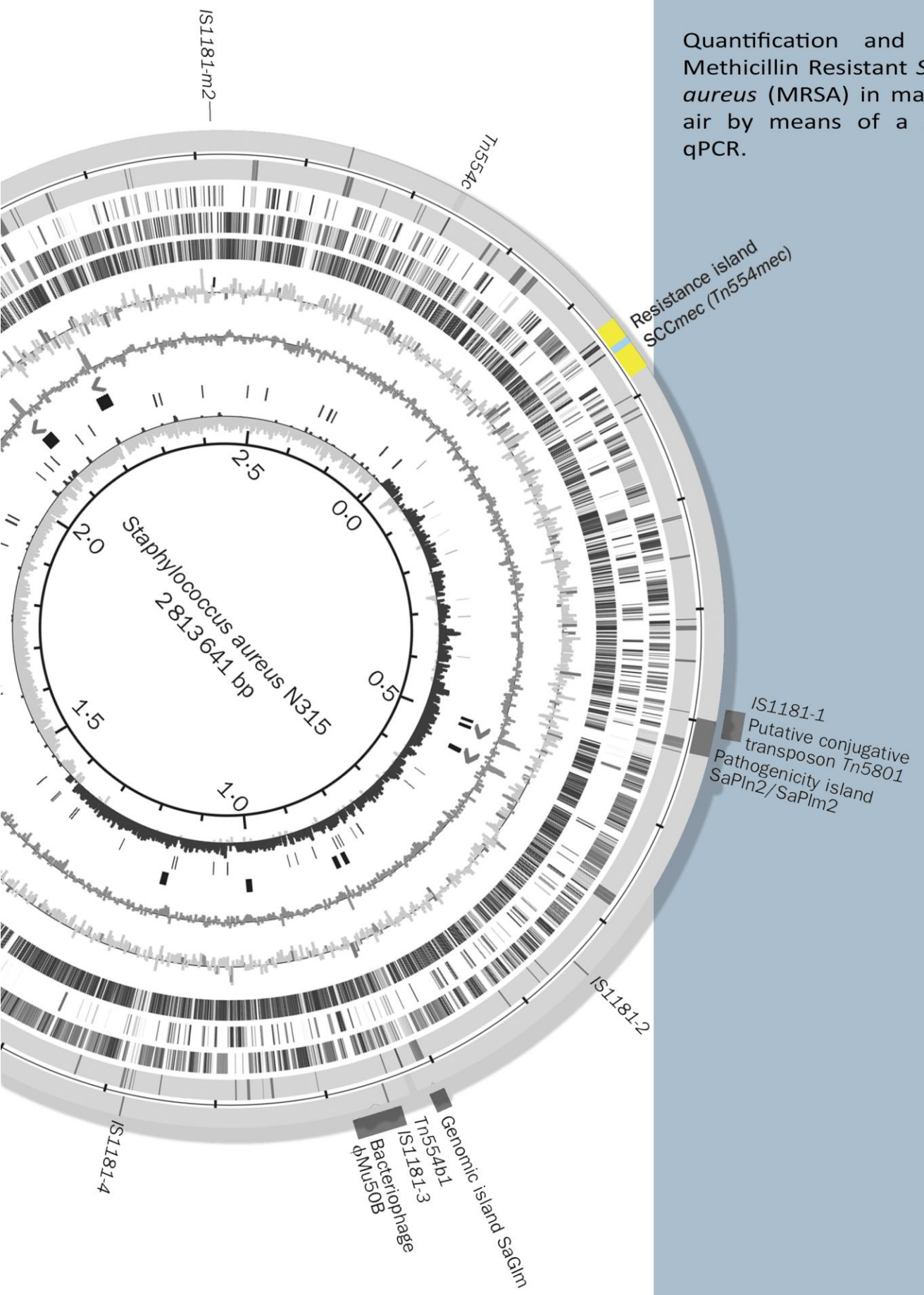
Research Internship

Quantification and detection of Methicillin Resistant *Staphylococcus aureus* (MRSA) in manure, soil and air by means of a *mecA* specific qPCR.



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Cover picture: Circular representation of *S aureus* chromosome (N315) and *Mu50*-specific genetic elements, SCC*mec* is marked in yellow, *Tn554mec* is marked in blue. (picture = edited) (Kuroda et al., 2001)

Content

Summary

Staphylococcus aureus is a gram-positive commensal and potentially pathogenic bacterium of many different animals as well as humans. Since 1961, *Staphylococcus aureus* gained resistance against methicillin (MRSA) and new clones arose, including the livestock associated MRSA ST398. The current research project focuses on the emergence and distribution of MRSA in and through the environment. More specifically, it focuses on the occurrence of MRSA ST398 in agricultural soil and air, in relation to soil fertilization, as already studied in the Dutch SKBII project by means of a ST398 qPCR. This study however, only detected limited amounts of MRSA ST398 in manure and in very limited amounts in soil and air. No direct relation between manuring and the occurrence of MRSA in soil and air could be established. As the *mecA* gene in MRSA defines its 'methicillin resistance', the current threefold research project focuses on the detection of this gene in manure, soil and air, in order to screen for wider (methicillin) resistance.

First a *mecA* specific qPCR was developed and optimized (Part I). Primers and probes were selected and several qPCR optimization steps were completed. Eventually a SYBR Green based assay was considered best suited for further research on *mecA* detection. Secondly, the detection limit of this particular qPCR was defined in relation to air filter samples. (Part II). Two different kind of filters (SKC and Pall) were spiked with serial decimal dilutions of different bacteria, including MRSA ST398. After DNA extraction, the amount of recovered cells per filter was calculated and compared with the initial amount of spiked cells per filter. The detection limit of both assays is situated somewhere between 1549-15488 cells per filter. The analytical sensitivity ranges from 33%-50% at a 10^{-5} (~1560 cells) spiking concentration and the regression analysis revealed a highly linear assay. Also, the recovered gene copies per qPCR, correspond rather well with the theoretically predicted amount of gene copies. The precision of both SKC and Pall assays is considered fairly modest, but could have been influenced by the removal of fluid during the extraction process. The SKC and Pall filter assays showed respectively 100% and 88% specificity, hinting towards possible assay contamination. The DNA extraction protocol, corresponding to the Pall filter assay, was found to be more suited to deal with higher bacterial concentrations. Some other comments can however be made about the assays accuracy. Not only is this study a laboratory-based assay, also no influence of real particulate matter (PM) on air filters was investigated. In addition, more cells were recovered from the filters, than spiked, which however also shows that the DNA extraction process didn't result in great DNA losses. Additionally, no inhibition testing was performed. Finally, the spiking technique itself should be redesigned for future detection limit testing. Part III of this report describes the detection of *mecA* in manure, air and soil samples derived from 16 Dutch pig, poultry and calf farms. However, no additional proof for the accumulation of antibiotic resistance was found in soil and air samples in comparison with the results from the MRSA ST398 qPCR assay (SKBII project). The results merely confirmed the low amount of antibiotic resistance (due to MRSA) in soil and air, except for the manure results from one farm, which indicated *mecA* presence not related to MRSA ST398. However, no indication for the transmission of antibiotic genes from manure to soil and air could be observed. As all samples were only tested with a qPCR, also death bacterial cells are included in the analysis, which do not contribute to the disease causing environmental bacterial load. Therefore, at this moment, no clear conclusions on specific health risks can be drawn from the findings in this report.

1. Introduction

Staphylococcus aureus is a gram-positive commensal and potentially pathogenic bacterium of many different animals as well as humans. (Verkade and Kluytmans, 2013, Deurenberg et al., 2007) In humans the colonization site most frequently observed is the nose – more specifically the vestibulum nasi – (average = 37.2% with a range of 19-55.1%) (Williams, 1963, Kluytmans et al., 1997), but also extra-nasal colonization is reported (e.g. skin, perineum, pharynx, the gastrointestinal tract, vagina and axillae). (Wertheim et al., 2005) A distinction can be made between persistent carriers (20% of the population), intermittent carriers (~60%) and noncarriers (~20%) and colonization can be induced by antibiotic administration. (Wertheim et al., 2005) In addition, carriage of the bacterium is a risk factor for developing infections. (Kluytmans et al., 1997) Infections in humans range from minor skin, surgical wound and soft tissue infections, to life-threatening disease conditions as bacteremia complicated by endocarditis, toxic shock syndrome and other metastatic infections. (Lowy, 1998) In livestock, infections most typically seen are associated with skin infections, infections of the urogenital tract, mastitis (typically seen in cattle) and joint disorders (mainly poultry). (Vanderhaeghen et al., 2010) Additionally, companion animals and horses can suffer from postoperative infections and wound infections with *Staphylococcus aureus*. (Leonard and Markey, 2008, Van Duijkeren et al., 2004)

1.1. Emergence of Methicillin resistance of Staphylococcus aureus (MRSA)

Penicillin resistance in *Staphylococcus aureus* was firstly discovered in 1942, when a penicillin resistant isolate was found in a human hospital. (Deurenberg and Stobberingh, 2008) In addition, two years after the introduction of methicillin in 1961, methicillin resistance was noted (Barber, 1961), after which resistance disseminated worldwide. (Vanderhaeghen et al., 2010, Brown et al., 2005, Deurenberg and Stobberingh, 2008) At first, MRSA was primarily related to nosocomial infections (Hospital-acquired/associated (HA) MRSA). (Deurenberg et al., 2007) In the 90s however, new non-healthcare related and genetically different, more virulent and moderate resistant – community associated (CA-MRSA) – clones were discovered. Their virulence is partly associated with the presence of Panton-Valentine leukocidin exotoxins (PVL) carried by certain CA-MRSA strains. (Zaidi et al., 2013, Deurenberg et al., 2007, Vanderhaeghen et al., 2010) During the period 1970-2000, MRSA was only sporadically isolated from animals and as such not considered a major zoonotic disease. (Catry et al., 2010) However, in July 2004, MRSA was isolated from a child living in a Dutch pig farming household (Voss et al., 2005), after which more data on livestock associated MRSA (LA-MRSA) was compiled and evidence on MRSA animal (mainly pigs, but also cattle and poultry) to human transmission was built, including in the Netherlands. (Huijsdens et al., 2006, Van Loo et al., 2007a, Crombé et al., 2013)

Staphylococcus aureus and MRSA can be typed by the use of multiple typing methods, including Multi-Locus Sequence Typing (MLST), *Staphylococcus aureus* protein A gene (*spa*) typing, Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing, Macro-restriction pattern analysis (PFGE) and Multi-locus Variable-Number Tandem Repeat Analysis (MLVA), with a recent trend towards the use of the first three methods. (Stefani et al., 2012) LA-MRSA is mostly found to be MLST sequence type (ST)398, belonging to clonal complex 398 (CC398). Yet, other STs are reported to be associated with livestock (i.a. ST9, ST97…). (Wagenaar et al., 2009, Crombé et al., 2013) At least 25 different *spa* types identified, belong

to this clonal complex including t(type)011, t034 and t108. (Vanderhaeghen et al., 2010) LA-MRSA is mostly associated with SCC*mec* types V, IVa (see also 1.2.), but also other types are reported. (Verhegghe et al., 2013, van Duijkeren et al., 2008, Wagenaar and Van De Giessen, 2009, Vanderhaeghen et al., 2010) A Dutch study, published in 2009, demonstrated a MRSA pig farm prevalence of 68,3% (98% ST398). Also calves (88% MRSA positive) and slaughterhouse broiler flocks (35% MRSA positive) were found to be mostly colonized with ST398. Dairy cattle were not sampled in this study. (Wagenaar and Van De Giessen, 2009) LA-MRSA is characterized by relatively few virulence associated factors (Crombé et al., 2013, Vanderhaeghen et al., 2010) – not precluding human and animal disease – and a relatively poor human to human transmission compared to non-MRSA ST398 genotypes (5.9 times less transmissible than non-ST398, as observed in Dutch hospitals). (Bootsma et al., 2011) MRSA strains also occur within companion animals, however, these strains differ from those in livestock and are suggested to be mostly acquired from pet owners (anthropozoonoses). (Morgan, 2008, Crombé et al., 2013) Literature mentions frequent resistance of ST398 strains against tetracyclines, but also resistance against macrolides, lincosamides, aminoglycosides, trimethoprim and fluoroquinolones has been listed. (Vanderhaeghen et al., 2010)

Transmission of MRSA ST398 from animals to humans occurs through direct contact or environmental contamination. (Verkade and Kluytmans, 2013) Until lately, LA-MRSA was not considered a major foodborne disease in the Netherlands. (Van Loo et al., 2007b). Yet, a recent Dutch case-control study indicated a significant relationship between the consumption of chicken meat and the carriage of CA-MRSA (including ST398). (van Rijen et al., 2013) Colonization of MRSA ST398 in humans is associated with the intensity of contact with livestock and the number of MRSA positive animals at the farm. Also, a higher risk exists for family members of a livestock farmer, if the farmer is a carrier. (Graveland et al., 2010) However, persistent carriage of ST398 has only been rarely demonstrated. (Van Cleef et al., 2011, Graveland et al., 2011) In addition, studies showed a positive relationship between antibiotic use and MRSA carriage among pigs and calves (van Duijkeren et al., 2008, Graveland et al., 2010), while farm hygiene is considered to be negatively correlated. (Graveland et al., 2010)

1.2. MRSA resistance depends on the expression of *mecA*

Genome sequencing of *Staphylococcus aureus* has revealed a complex mixture of genes with evidence of acquisition trough lateral gene transfer. (Hiramatsu et al., 2001) Resistance against β-lactam antibiotics in *Staphylococcus aureus* is mediated by the bacterial production of β-lactamase (encoded by *blaZ*) – which inactivates penicillin by hydrolysis of its β-lactam ring – and the presence of penicillin binding protein 2a (PBP2' or PBP2a). PBPs are transpeptidases responsible for catalyzing the formation of cross-bridges during the peptidoglycan synthesis of the bacterial cell. PBP2', however, is a 78 kDa aberrant PBP coded by *mecA,* with a low binding affinity for β-lactam antibiotics, permitting cell wall synthesis even at clinically relevant antibiotic concentrations. (Zhang et al., 2001, Hiramatsu et al., 2001) *MecA* itself, is located at the 3' end of an open reading frame (*orfX*) on acquired mobile genetic element 'Staphylococcal Cassette Chromosome *mec*' (21-67 kb) (SCC*mec,* an antibiotic resistance island). *MecA* is part of a *mec* gene complex together with its regulatory genes (e.g. *mec*R1 and *mec*I) and associated insertion sequences. A 'SCCmec type' is also determined by the cassette chromosome recombinase gene complex (*ccr*; responsible for

excision and integration in the chromosome of SCC*mec*) and Joining regions (J region; non-essential components of the cassette). (Hiramatsu et al., 2001, IWGCSCCE, 2009) Expression of methicillin resistance is nevertheless heterogeneous between and within populations and depends on the presence of multiple gene elements as e.g. *mecI* (encoding a transcription repressor protein), the β-lactam-sensing signal transducer *mecR1*, certain housekeeping genes as *fem* (peptidoglycan-modifying enzymes), *aux* (auxiliary factors), the possible co-regulation by the β-lactamase induction system and many more elements. (Berger-Bächi, 1994, Berger-Bächi and Rohrer, 2002, McCallum et al., 2010, De Lencastre and Tomasz, 1994). *Staphylococcus aureus* can also harbor other resistance genes inside and outside the SCC*mec* region (e.g. inside: SCC*mec* types II and III incorporate plasmids and transposons carrying resistance). (Deurenberg and Stobberingh, 2008)

The *mecA* gene and SCC*mec* elements are not exclusively present in MRSA. Moreover, it has been proposed that *Staphylococcus fleuretti* has served as the *mecA* origin for MRSA. (Tsubakishita et al., 2010) In the environment, other methicillin resistant coagulase negative staphylococci (MRCNS) as *S. fleuretti* harbor SCC*mec* elements. MRCNS carriage has been demonstrated in humans and animals (e.g. livestock (Vanderhaeghen et al., 2012, Huber et al., 2011, Bagcigil et al., 2007) and companion animals (Bagcigil et al., 2007, Van Duijkeren et al., 2004)) and horizontal transfer of *mecA* among MRCNS in production animals can occur. (Huber et al., 2011) Due to the fact that SCC*mec* elements – that are predominant in MRSA ST398 – are discovered in MRCNS in pigs and that *in-vivo* horizontal transfer between MRCNS and MRSA has also been observed (Wielders et al., 2001), it has been suggested that these bacteria could serve as a potential reservoir for MRSA in animals (e.g. in pigs (Tulinski et al., 2012, Vanderhaeghen et al., 2012)). Finally, the *mecA* gene has also been detected in non-staphylococcal microorganisms (*Enterococcus faecalis*, *Proteus vulgaris* and *Morganella morganii*; all intestinal bacteria of humans and other mammals) (Kassem et al., 2008), hinting towards broader horizontal transfer of the *mecA* gene among different bacteria.

1.3. MRSA resistance in the environment

Resistance results from selection and mutation caused by e.g. antibiotics, xenobiotics or heavy metals, but also through horizontal transfer (e.g. transformation, transduction or conjugation). (Gaze et al., 2008) In recent years, new insights were gained on the transmission of MRSA ST398 through the environment, including the transfer of resistance genes.

Firstly, antibiotic resistance genes and plasmids are proven to be present in manure. (Binh et al., 2008) Moreover, the occurrence of antibiotic resistance in manure is recently reviewed and a various group of studies have reported correlations between manure application and the occurrence of resistance genes in surface water and even fertilized soils. (Heuer et al., 2011) Secondly, studies found evidence for the presence of airborne MRSA ST398 and its resistance genes in association with Dutch livestock farming. (Heederik, 2011) In addition, studies indicated MRSA ST398 airborne spread within pig herds and in the vicinity of pig barns. (Schulz et al., 2012, Friese et al., 2012) Also airborne transmission from Dutch slaughterhouse pigs to humans through air was suggested and working in the lairage, scalding or dehairing area of the slaughterhouse was considered a risk factor. (Gilbert et al., 2012)

As horizontal transfer between staphylococci – and between staphylococci and other bacteria – plays an important role in the transfer of antibiotic resistance (Gaze et al., 2008, Allen et al., 2010), it is safe to assume that this could also occur between MRSA and indigenous soil bacteria. This fact, but more importantly the emergence of MRSA and MRSA ST398 in animals and consequently in manure, can possibly create a public health risk for people in contact with fertilized soil, its water drainage and even inhaled dust of whirled soil.

2. Aim of the current research project

The subsequent research is part of a larger research project on the presence of antibiotic resistant bacteria (MRSA, Extended Spectrum Beta-Lactamases (ESBLs)) in agricultural soil and air before and after soil fertilization. The aim of this particular research will be discussed after a brief presentation of the main research project with a focus on MRSA.

2.1. The umbrella research project – Aim and outcome

One part of the overarching research project ('Antibiotic resistance in soil part II' – 'Stichting Kennisontwikkeling Kennisoverdracht Bodem II (SKBII)') was to define the relationship between manure application and the occurrence of antimicrobial resistance in arable soils and air (aerial drift). This study succeeded a Dutch pilot study which detected an increase of resistance genes (*tetM* and *sul2*) at three farm field locations after fertilizing. Briefly, during the SKBII project, samples were taken from 16 different farms – that participated on a voluntarily basis – before (= t1) and two weeks after (= t2) fertilization of their farmlands. These samples included field soil samples (t1 and t2), samples from the manure pit (t2) and air samples (depending on the farm t1 and/or t2). Soil samples consisted of soil acquired from different locations in the field (3 times 20 samples on two straight lines) and were taken from the top (0,5 cm topsoil) and of a deeper layer (10 cm depth) of the fields. Mixtures of these samples were made (subplot samples) until 6 samples remained (3 of topsoil, 3 of 'deeper layer soil'). Air samples (Gillair pump 3,4 L/min during 6h and 37mm Teflon filters, 2µm pores, Air Diagnostics and Engineering Inc, Naples, ME, USA) were taken upwind (1^{st}), 5m upwind within (2^{nd}) and in the middle (3^{rd}) of the field. One sample (4^{th}) was taken downwind (5m within) the field. Analysis of the samples comprised of cultivation (mixed samples from top and 'deeper layer' soil in combination with qPCR on MRSA ST398 and MALDI-TOF on coagulase-positive strains) and qPCR analysis (*16S* and ST398). Also questionnaires were conducted in relation to the nature of the farm, its manure application and antibiotic use. The results of this study however, turned out to be negative concerning the cultivation of the soil samples. On the contrary ST398 qPCR results showed that some manure samples were positive – although only slightly – and that those were mainly from pig farms. In addition, 4 of 192 soil samples and some air samples indicated ST398 presence but also in very modest amounts. For more details on the sampling process and the outcome of this study, the reader is referred to: (Schmitt et al., 2013).

As manure, soil and air samples were only specifically tested with a ST398 qPCR, the question remained if other MRSA genes or MRSA resistant strains could be detected. As described before, *mecA* codes for methicillin resistance and consequently can be used to test for MRSA. Detection of *mecA* by means of PCR has been described as the most robust and reliable way of detecting oxacillin-resistant staphylococci. (Fluit et al., 2001) The detection of *mecA* is even considered the gold standard method in comparison with culture-based methods, mainly because phenotypic methods may be difficult to interpret and because some isolates do not express their *mecA* gene unless selective pressure via antibiotic treatment is applied. (Martineau et al., 2000) The latter remark is especially important. It means that methicillin susceptible strains (MSSA) containing the *mecA* gene should be regarded as potentially resistant and thus not merely as false-positive results. This statement is based on the previously demonstrated ability of *in vitro* selection of resistant cells from this kind of susceptible strains, by the use of plates with an increasing antibiotic

concentration gradient. (Martineau et al., 2000) Nevertheless, the fact that *mecA* can also be found in MRCNS and possibly in non-staphylococcal microorganisms, results in less specificity when using *mecA* as the principal target in a qPCR to identify MRSA in highly diverse ecosystems as arable soils. Recently, also a new drawback to the use of *mecA* qPCR was described. The sensitivity of these molecular assays to detect MRSA was questioned, when a ß-lactam resistant *S. aureus* strain isolated from bulk milk (from cattle) –carrying a genetically different *mecA* homologue (*mecA*$_{LGA251}$, type-XI SCC*mec*) – was described. (García-Álvarez et al., 2011) This *mecA*$_{LGA251}$ was detected in humans and cattle and its prevalence was estimated to be in the range of 1 in 100 to 1 in 500 of total MRSA in the UK and Denmark. This *mecA* homologue could not be detected with conventional PCR for *mecA*, owing to the different nucleotide composition (70% nucleotide homology with *mecA*). The use of *mecA* qPCR therefore, can also be prone to false-negative results, especially when at this moment more and more *mecA*$_{LGA251}$ animal reservoirs are being indentified. (Petersen et al., 2013)

No MRSA ST398 qPCR detection limit (LOD) was defined for soil and air samples during the SKBII study, because the majority of the samples were negative. According to Burns et al. (2008), one of the most important reasons for defining the LOD is to identify where the method performance becomes insufficient for acceptable detection of the target analyte, in order that subsequent analytical measurements can stay away from this problematic area. qPCR measurements around the limit of detection are therefore i.a. more prone to and are hampered by noise, an unstable baseline, losses during the DNA extraction, isolation or even the cleanup process. Especially when low level data is reported and when no additional culturing is performed (often better able to detect low level MRSA, in particular when selective enrichment is used (Brown et al., 2005)), the qPCR LOD makes the reader aware of the limitations of the concerning data.

Therefore, the present research project is threefold. First a *mecA* specific qPCR is developed (Part I), subsequently the detection limit of this qPCR is examined in relation to air filter samples (Part II) and finally the 'SKBII samples' are tested and analyzed (Part III).

3. Part I: Development and optimization of a *mecA* qPCR

3.1. Part I: Materials and methods

3.1.1. Selecting, culturing and DNA extraction of two MRSA strains

Two *Staphylococcus aureus* strains were selected in order to optimize the qPCR and to design a calibration curve: an unknown MRSA strain (probably ST398 – 03-2013) and MRSA AP009324 (*tetM+*, 31-10-2008), from here on respectively referred to as 'MRSA unknown' and 'MRSA *tetM+*'. The bacteria were cultured on trypticase soy agar (TSA) and incubated one day at 37 degrees Celsius (°C). The next day cultures were streaked on TSA and incubated at 37°C overnight, after which three tubes of trypticase soy broth (TSB) were inoculated with one colony of each bacterium – 3 tubes per bacterium – and put away at 37°C for 24 hours.

Three different protocols were used for the extraction of DNA from the six TSB tubes and are described in detail in Annex I. The application of these protocols resulted in 18 different DNA samples (3 protocols, 3 replicates (= 3 TSB tubes), 2 bacteria). The first replicate of each protocol and bacterium was tested and analyzed with NanoDrop®2000 (t0) and the calculated absorbance ratios (260/280: measure of purity of dsDNA and 260/230: secondary measure of dsDNA purity) were assessed. (Desjardins and Conklin, 2011)

3.1.2. Selection of the qPCR primers

A literature study was held in order to systematically search for studies on *mecA* (q)PCRs. 20 studies concerning original *mecA* PCRs were considered eligible and a database was created in which details of the selected studies and the applied primer pairs were listed, including length of the primers, amplicons and probes, references to GenBank accession numbers (National Center for Biotechnology Information (NCBI)), the use of environmental or clinical samples for primer testing and how many times the study was cited. Primers were also evaluated based on GC content, location of the specific Gs and Cs, the published qPCR protocols, and if mentioned, the related sensitivity and efficiency data. After initially ranking and excluding the first primers, the primer pairs still in the running were evaluated with ClustalX2.1 (check for fitting), Primer-Basic Local Alignment Search Tool (Primer-Blast, NCBI; check for cross-reactivity) and Mfold Web Server (check for secondary structures). If a specific primer pair was found suitable, but wasn't already used in a qPCR and consequently not associated with a probe, other probes of other eligible primer pairs were checked for suitability. Finally, three primer pairs with associated probes were selected for the development of a *mecA* qPCR.

3.1.3. Details of the qPCR reactions

SYBR Green assays were performed using the iQ™ SYBR® Green supermix (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) containing SYBR® Green I dye, 50 U/ml iTaq™ DNA polymerase (= hot-start), dNTPs (0.4 mM each of dATP, dCTP, dGTP and dTTP), 6 mM $MgCl_2$, 40 mM Tris-HCl, pH 8.4, 100 mM KCl, 20 nM fluorescein, and to the author unknown stabilizers. Probe conducted assays containing a probe were held with iQ™ Supermix (Bio-Rad) containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP

(dATP, dCTP, dTTP), 50 U/ml iTaq DNA polymerase (= hot-start), 6 mM $MgCl_2$ and to the author unknown stabilizers. Mastermix preparation was carried out in the 'PCR-laboratory' in a laminar flow cabinet, after which template was added in a separate laboratory. Assays were performed on a CFX384™ Real-Time detection system (Bio-Rad) with a total volume of 10 µl per reaction (3 µl template, initial DNA concentration of 0.001%). Every sample was at least run in triplicate and non-template controls (NTCs) consisted of mastermix with Milli-Q water (MQ) as 'template' – including MQ used during the mastermix preparation and MQ used for the preparation of DNA dilutions. Oligonucleotides were ordered from Eurogentec, Seraing, Belgium.

3.1.4. qPCR validation parameters

The optimization process in duplo (MRSA unknown, MRSA *tetM*+) consisted of several steps including the running of a qPCR gradient, testing multiple primer and probe concentrations, the use of positive and negative environmental samples and the testing of two separate calibration curves. In general, all qPCRs were run for 40 cycles. Also the use of a two-step versus a three-step qPCR protocol was considered, although the latter was regarded unnecessary in the end, as the two-step protocol already performed well.

In order to further judge the above mentioned steps, certain validation parameters were used e.g. analytical sensitivity/linearity and efficiency, analytical specificity, precision and accuracy. Ruggedness (robustness) – the ability of a method to resist changes in results when subjected to minor changes in environmental and procedural variables, laboratories and personnel – was tested by the above mentioned gradient and primer/probe concentrations steps. (Lipp et al., 2005) No inter-laboratory and inter-personnel variation was investigated.

Analytical sensitivity refers to the minimum number of copies in a sample that can be measured accurately with an assay. (Bustin et al., 2009) However, during the optimization process, the sensitivity of the assay – more specifically of the calibration curve – was defined by the dilution before the dilution (from threefold dilutions) at which the assay no longer functions in a linear fashion with the higher template concentrations. (Shiley, 2011) Standard deviations of the three qPCR replicates per sample do not exceed 0,5 C_q. Analytical specificity – the ability to specifically amplify and detect the appropriate target sequence (Bustin et al., 2009) – was evaluated by gel electrophoresis (UltraPure Agarose™, 100 bp DNA Ladder and SYBR® Gold Nucleic Acid Gel Stain, Invitrogen, Bleiswijk, The Netherlands) and melting curve analysis (Bio-Rad software). Specificity was also enhanced in this experiment by the inclusion of probes during the optimization process and by using a hot-start DNA polymerase as described above. Finally, also MRSA positive and negative manure samples were tested to check for specificity.

Efficiency was calculated using the slope of the calibration curve as presented in the following formula:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1.$$

The minimum efficiency of the initial calibration curve had to fall within the range of 90-110% (100%, slope = -3.32; reflecting doubling of qPCR product after each cycle). However, also values starting from 85% were accepted. Also the coefficients of determination (R^2) were judged and were considered to be at least 0,980. (Pestana et al., 2010)

Precision describes the degree to which repeated measurements under unchanged conditions show the same results (Hospodsky et al., 2010) and can be divided in repeatability (short-term precision or intra-assay variance) and reproducibility (long-term precision or interassay variance). (Bustin et al., 2009) During the optimization process only short-term precision was evaluated by comparing the standard deviations of the intra-assay C_qs among technical replicates (SD < 0,5; minimum of three replicates per sample). Also calibration curves of the different assays (including assays from Part II and III of this report) were compared by means of the coefficients of variation (CVs). Accuracy is expressed as the degree of closeness to the true value. (Hospodsky et al., 2010) Here it was controlled by running NTCs, the designing of two separate calibration curves and by testing MRSA positive and negative manure samples (samples from the 'Bactopath project', IRAS, Utrecht University, The Netherlands: unpublished data).

3.2. Part I: Results

3.2.1. Results DNA extraction

In total 2,55ml DNA was extracted of each bacterium. Based on the absorbance ratios generated by NanoDrop™ 2000 (260/280 and 260/230), DNA extraction protocol two delivered the highest DNA concentration, however, this DNA showed the highest degree of contamination (e.g. with EDTA, phenols, proteins etc.). The absorbance ratios of the DNA samples generated by protocol one and three lied close to one another (Table I). The decision to select the DNA obtained through protocol one, was ultimately based on the clearest DNA peak (Figure I) reflected in the highest 260/280 ratio.

Table I: Results of the NanoDrop™ 2000 software analysis of the extracted DNA from MRSA unknown and MRSA *tetM+*.

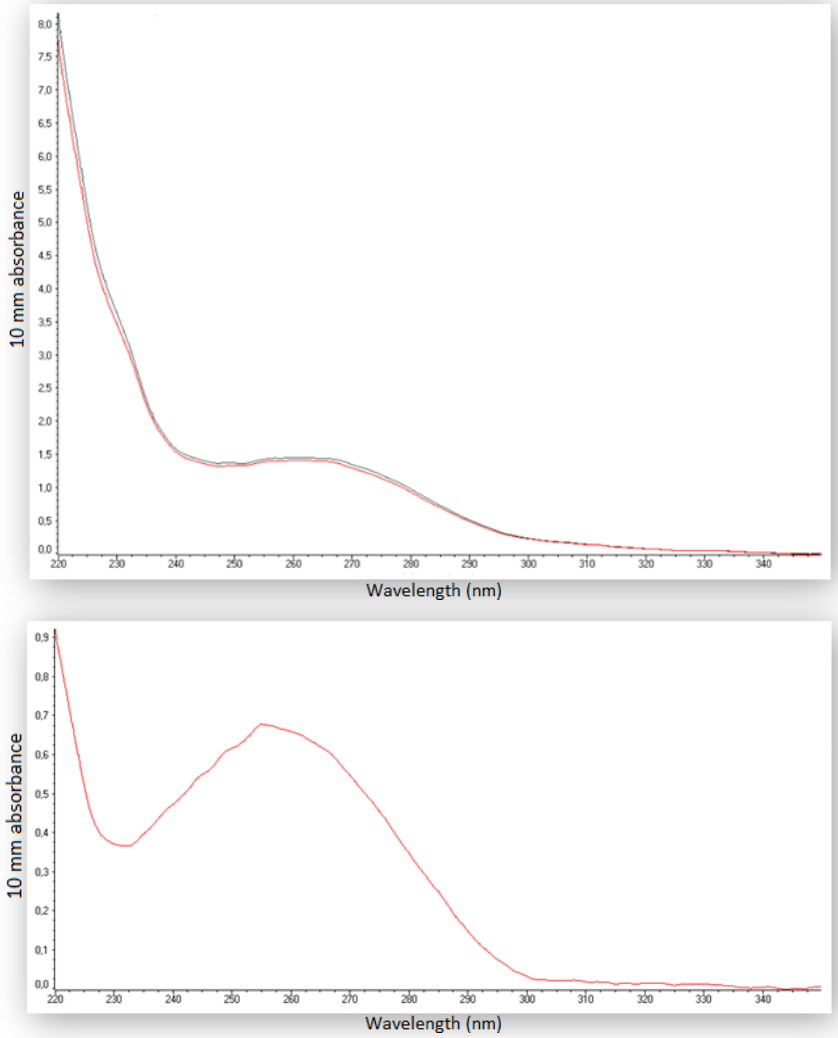
Sample	Point in time	Protocol	DNA concentrations (mean, ng/µl)	260/280 (mean)	260/230 (mean)
MRSA unknown	t0	1	76	1.39	0.34
MRSA *tetM+*	t0	1	102	1.39	0.36
MRSA unknown	t0	2	551	0.56	0.18
MRSA *tetM+*	t0	2	580	0.56	0.19
MRSA unknown	t0	3	65	1.25	0.62
MRSA *tetM+*	t0	3	80	1.3	0.65
MRSA unknown	t1	1	70	1.52	0.4
MRSA *tetM+*	t1	1	72	1.52	0.39
***MecA* qPCR product**	NA	NA	34	1,89	1,84
Absorbance ratios pure DNA	NA	NA	NA	1,8	2 - 2,2

Key: NA = not applicable

All solution replicates of DNA extracted with protocol one, were added together, the DNA concentration was redetermined (t1) and run on a control agarose gel.

During the course of the research process (mainly Part II) an overestimation of the initially calculated DNA concentration was suspected. Following this, it was decided to purify (protocol in Annex II) amplified PCR product generated from the initial calibration curve (MRSA unknown). The purified DNA was evaluated by NanoDrop™ 2000 and a new calibration curve was prepared.

Figure I: Spectral images generated by the NanoDrop™ 2000 software: Comparison initially extracted DNA (top: MRSA unknown, red; MRSA *tetM*+, black) and purified qPCR product (bottom).



As seen in Table I and Figure I, the purified DNA has a much more clear DNA peak and higher absorbance ratios in comparison with the initially extracted DNA.

3.2.2. Results of the primer selection process

Only two primer pairs ('*Pasanen*' - (Pasanen et al., 2010) and '*François*' (Francois et al., 2003)) met the basic criteria and showed no abnormalities when using ClustalX2.1, Primer-Blast® and Mfold Web Server®. Additionally, one extra primer pair '*Sabet*' (Sabet et al., 2007) was selected. However, no sensitivity and efficiency data of this assay was mentioned in the

corresponding article. In addition, the primer pair was not tested on environmental samples, but scored positive on all other criteria. All selected primer pairs are listed in Table II. A table mentioning all analyzed primer pairs, can be found in Annex III.

Table II: Selected primer pairs and associated probes.

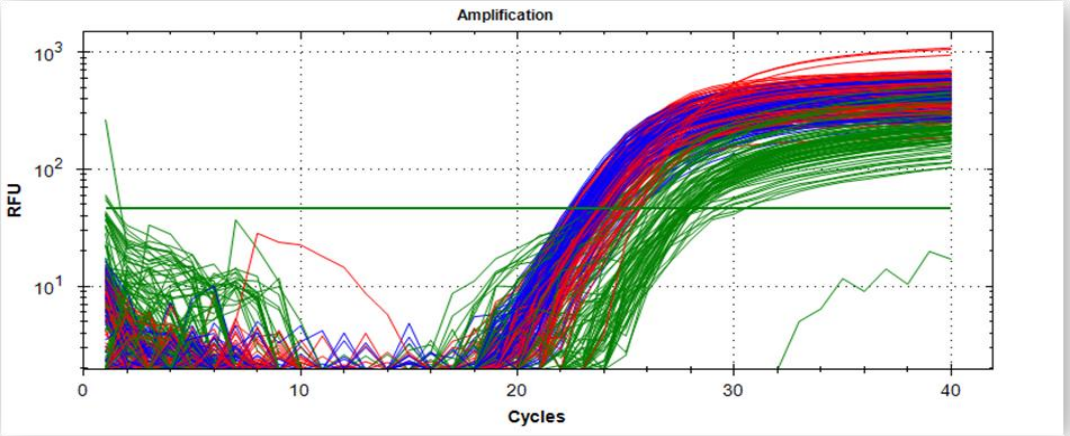
Reference	*Fw* (5'-3')	*Rv* (5'-3')	Probe (5'-3')	Amplicon length
(Francois et al., 2003)	CATTGATCGCAACGTTCAATTT	TGGTCTTTCTGCATTCCTGGA	FAM-TGGAAGTTAGATTGGGATCATAGCGTCAT - BHQ1	99
(Pasanen et al., 2010)	GATTATGGCTCAGGTACTGCTATCC	ATGAAGGTGTGCTTACAAGTGCTAA	VIC-CCCTCAAACAGGTGAATT - BHQ1	70
(Sabet et al., 2007)	AAAACTAGGTGTTGGTGAAGATATACC	GAAAGGATCTGTACTGGGTTAATCAG	FAM-TTCACCTTGTCCGTAACCTGAATCAGCT - BHQ1	147

Key: *Fw*: forward primer, *Rv*: reverse primer.

3.2.3. Optimization *mecA* qPCR

Three gradient qPCRs (200nm primer starting concentration) were run to test the three primer pairs at 16 different annealing temperatures (T_as) between 66.7°C and 54.7°C (second gradient range 55.6-65.6°C). Overall, these gradients showed that primer pair *Pasanen* was slightly less sensitive (i.c. higher C_q values) than primer pairs *François* and *Sabet* when comparing the lowest C_qs (mean difference: 0.62-1.02 C_q). Regarding this observation, it was decided to discard primer pair *Pasanen* from the rest of the experiments. Optimum T_as for primer pair *François* were twofold and were established between 58.8-60°C and between 61.4-62.7°C (preferably around 60°C). Optimum T_as for primer pair *Sabet* are around 62.7-63.9°C (preferably around 63) and 58.8-60°C. Optimum melting temperatures (T_ms) were found to be 76.5 (primer pair *François*) and 76 (primer pair *Sabet*). No abnormal melting peaks were observed. As primer pair *François* showed slightly more sensitivity, also the associated probe was tested in a gradient qPCR. Results showed an optimum primer/probe temperature around 58.8-60°C and lower sensitivity, even at high DNA concentrations (Figure II). The performance of the probe, associated with primer pair *Sabet*, was not yet evaluated due to the low T_a (55°C) of this probe reported in the corresponding study. This T_a might not match the high T_a (63°C) of the primers found during the gradient qPCRs.

Figure II: Gradient qPCR showing amplification of MRSA unknown and MRSA *tetM*+.



Key: Curves: blue = primer pair *François*, green = primer pair *François* with associated probe, red = primer pair *Sabet*. The relative fluorescence units (RFU) are presented on a log scale.

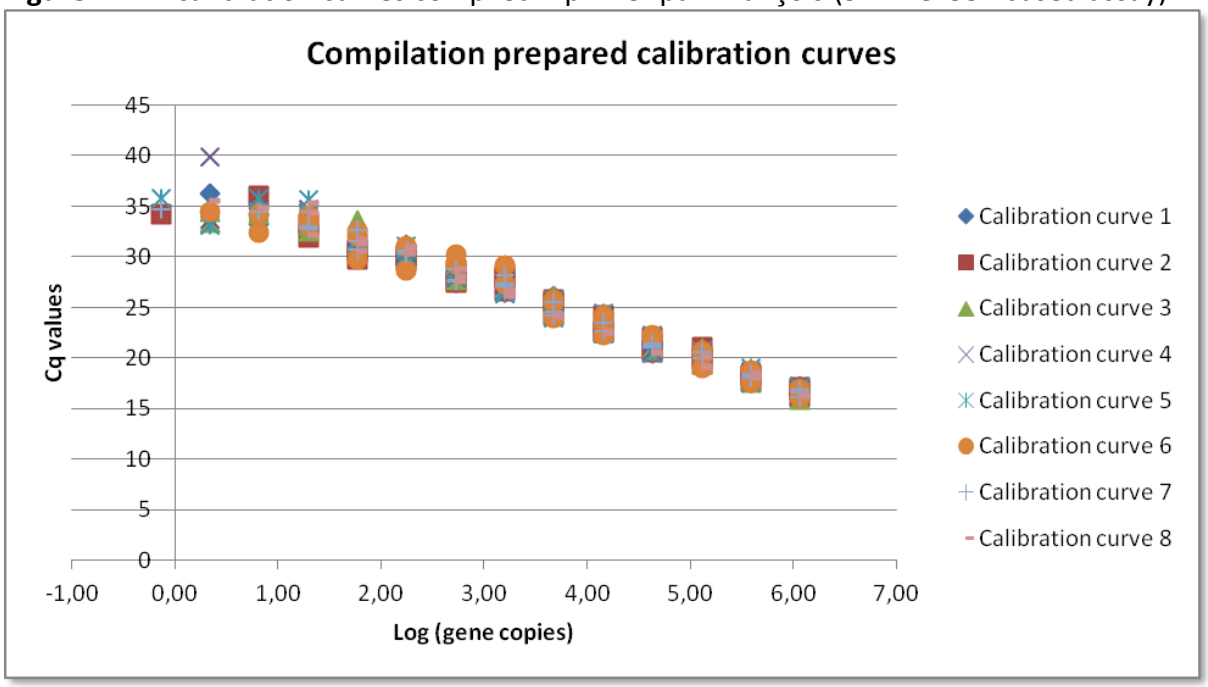
Concentrations of the primer pairs *Sabet*, *François* (100, 200 and 300nm) and probe *François* (100 and 200nm), were tested at a T_a of 60 and 63°C (the latter: only primer pair *Sabet*). 200 and 300 nm primer concentrations performed evenly well and gave the best results. Furthermore, at the finally chosen 200nm primer concentration (primer pair *François*), a 200nm probe concentration was considered suitable, although a concentration of 100 to 200nm shows a little bit more sensitivity. MRSA positive and negative manure samples were tested and as such, it was found that the assay with probe detected more positive samples (however only when running 50 instead of 40 cycles). It should be noted on the other hand, that certain so-called negative samples were found positive by all assays (primer pair *François* with and without probe and primer pair *Sabet* without probe), which should be noted as non-specificity. It was also found that all qPCRs held with primer pair *Sabet* were prone to nonspecific product formation in the non template controls, which also became apparent when testing the manure samples. These observations led to the withdrawal of this primer pair from further testing.

Three calibration curves were prepared using MRSA unknown, MRSA *tetM*+ and the purified PCR product as mentioned above. The initial calibration curves using the two different bacterial strains were found to agree well. Dilutions (1/3 and 1/10) started from the undiluted DNA until a concentration of around one expected *mecA* gene copy. The calibration curve prepared with amplified PCR product, was generated from a probe based assay (primer pair and probe '*François'*) and compared with the initial 'MRSA unknown' calibration curve, generated from a similar probe based qPCR assay. This comparison revealed a 6 times underestimation of the initial DNA concentration. As, at this point, already all assays from Part II and Part III were performed, consequently all results from the SYBR Green based assays had to be corrected with a correction factor (mean value = 6,2). In contrast, the outcomes of the probe based assays were merely related to the newly generated calibration curve. From here on, only corrected data is presented.

Efficiencies of the assays with primer pair *François* (with and without probe) were comparable (mean: respectively 89 versus 93%). The R^2s of the SYBR Green based assays were nearly always in range in contrast to the assays with probe (mean: 0.976), partly due to

a pipette calibration error which affected the probe based assay the most.[1] The calibration curve of the assay without probe showed linearity starting from dilution 3^{-2} (clear inhibition before this point) representing 1,16E+06 gene copies until dilution 3^{-11} (or 10^{-5}) with a mean C_q of 32,5 representing ±59 gene copies (105 gene copies in the 1 in 10 dilution series) with nearly all technical replicates being positive (Figure III). The assay with probe was linear until a C_q of 35, representing the same amount of absolute gene copies as the assay without probe.

Figure III: All calibration curves compiled – primer pair *François* (SYBR Green based assay).



Key: Calibration curves were generated using the initially estimated DNA concentration (refer to 3.2.1) and were corrected with a correction factor, when it was discovered that these concentrations were overestimated (data exported at threshold 40,5).

As seen in Figure III, the C_qs from the different calibration curves (per log gene copy) vary strongly among each other. This variation can probably partly be explained by the already mentioned pipette calibration error only discovered later in the experiment (Paired student T-Test – $P<0.001$; based on the different coefficients of variation before and after correction of the calibration error).

The limit of quantification (at 95% detection) was calculated at the C_q cut-off of 32,5 (assay without probe) and 35 (assay with probe) which corresponds respectively to 86,10 (1,94 log gene copies, $P<0.001$) and 69,10 (1.84 log gene copies, $P=0.639$ and therefore should only be expected with 95% certainty between 19.71 and 177.38 gene copies) gene copies. (N.B. Figures are calculated among the C_qs of all qPCR replicates per gene copy number.)

[1] During the qPCR mastermix preparation, an electronic 8 channel VIAFLO Voyager Pipette (INTEGRA) was used. It was however noticed that during the first dispense step – after aspiration of mastermix containing the primers and optional probe – not as much fluid was released compared to the following dispense steps. This error has caused variation in qPCR signal among the different wells. This error had a larger impact on the assays with probe, because relatively more SYBR Green than necessary was used during the assays, in comparison with the more carefully calculated amount of probes.

3.3. Part I: Discussion and conclusion

Finally primer pair *François* was chosen for application during further research. The following protocol was designed:

Protocol I: SYBR Green assay
Primer pair: *François.*
Primer concentrations: 200nm.
Protocol: 3' at 95°C; 15" 40x 95°C; 60" at T_a 60°C.
Melting curve analysis is necessary (T_m: 76,5-77).
The amplified PCR product needs to be run on agarose when the associated $C_q \geq C_q$ cut-off (32,5), as samples with high C_qs sometimes showed good melting peaks, but presented incorrect bands on gel.

The efficiency of the assay was at least 90%, with a dynamic range from 3^{-2}-3^{-11} (around 59 gene copies) while showing good accuracy, precision and robustness. One remark should however be made. As certain presumably negative environmental (manure) samples were found to be positive after application of the different assays, certain doubts were cast on the specificity of the assay.

In the initial article (Francois et al., 2003), the *mecA* gene was tested as part of a triplex probe based qPCR (*mecA*, *femA* from *S. aureus* and *femA* of *S. epidermidis*), possibly explaining the lower primer and probe concentrations used during this multiplex assay, namely 100 nM (primers) and 75 nM (probe) in a 20µl final PCR mixture. Although the authors mention a linear dynamic range from 10^6 gene copies until 1-2 gene copies (slope -3.59, $r^2 > 0.99$), a graph from the regression analysis (representing *mecA* C_qs with respect to log gene copies) however, demonstrates deflection of the curve at a C_q of 35 and larger standard errors of the mean starting from 100 gene copies. These findings are in line with the current probe based assay (although a singleplex assay). Also the optimal T_a found during the current research project was the same as the T_a used by the authors.

In the end the application of the primer pair *François* in a probe based assay didn't show as much precision and sensitivity in comparison with the assay without probe, especially within a maximum of 40 qPCR cycles. A probe in general however, offers more specificity and a high signal to noise ratio. (Pestana et al., 2010) Consequently, a SYBR Green based assay could be more prone to overestimation in relation to the amount of gene copies in a given sample. Because of this fact, samples from Part II and III of this project, were also tested with the probe based assay for general comparison (the protocol is illustrated below). Yet, the SYBR Green based assay was always considered leading.

Protocol II: Probe based assay
Primer pair: *François.*
Primer concentrations: 200nm.
Probe concentration: 200nm is suitable, but lower concentrations (100-150nm) result in slightly higher sensitivity.
Protocol: 3' at 95°C; 15" 40x 95°C; 60" at T_a 60°C.
Control of the amplicon on an agarose gel is recommended, especially at high C_qs.

4. Part II: Detection limit study

4.1. Part II: Materials and methods

4.1.1. Preparation of the cultures

5 bacterial strains were chosen for cultivation: MRSA ST398 (August 2012), *Escherichia coli* (*E. coli*) CTX-M1 (*Xu*) pGEM®–T Easy (November 2011), *E.coli tetW* pGEM®–T Easy (August 2010), *E. coli intI1* pGEM®-T Easy (March 2012) and *E.coli* American Type Culture Collection (ATCC) 25922 (already kept at the laboratory refrigerator at 4°C in TSB). The different GMO's (*E. coli* CTX-M1, *tetW* and *intI1*) contain plasmids, carrying different genes related to antimicrobial resistance, and were included for future detection limit testing on these genes. *E.coli* ATCC was incorporated, in order to adjust total numbers of spiked bacteria. *E.coli* CTX-M1, *intI1* and *tetW* were grown overnight in Tryptic Soy Broth (TSB) containing ampicillin 100µg/ml at 37°C and were afterwards held at 4°C during 2 days. Following these two days, also a MRSA ST398 pure culture was obtained by using the streak plate method on Tryptic Soy Agar (TSA) containing 4 mg/l oxacillin, in order to initially select for oxacillin resistant *Staphylococcus aureus* strains. Additionally, TSA containing 100µg/ml ampicillin was inoculated with TSB containing the cultured GMOs. Furthermore, *E.coli* ATCC was streaked directly on TSA from the inoculated TSB at room temperature. All plates were first kept at 37°C overnight and at 4°C during 24h. Hereafter, TSB with ampicillin was again inoculated with one colony of the different GMO's. TSBs with and without oxacillin were inoculated with one MRSA ST398 colony from the TSA and plain TSB was used for one colony of *E.coli* ATCC. All tubes were then kept overnight at 37°C.

4.1.2. Spiking of the filters

Two filter types were chosen for spiking: SKC filters (PTFE (polytetrafluoroethylene), 37 mm, 2µm pore size – SKC Inc. Pennsylvania, USA) and Pall filters (PTFE, 37 mm, 2µm pore size Pall® Life Sciences, Michigan, Ann Arbor, USA). These filters were aseptically placed in sterile 15 ml Greiner tubes. The tubes were labeled and decimal dilution series of the inoculated TSBs containing the different bacteria (MRSA ST398 (only the TSB containing oxacillin), *E.coli* CTX-M1, *E.coli tetW*, *E.coli intI1* and *E.coli* ATCC) were prepared, ranging from +1 (= 10 times the original concentration) to -7 (dilution 10^{-7}). The highest spiking concentration was achieved by using a higher volume addition of undiluted TSB. More details regarding the different spiking concentrations and the number of filter replicates spiked, can be found in Annex IVa+b. The interval between inoculation and spiking was approximately 20-23hrs. After the spiking process, tubes containing the spiked filters were instantly frozen – using liquid nitrogen – and stored at -80°C until DNA extraction.

While spiking the filters, 100 µl of the different dilutions were plated on TSA in quadruple (MRSA ST398: dilutions -3 to -6; *E.coli* ATCC: dilutions -4 to -7) and TSA containing 100µg/l ampicillin (GMOs: dilutions -4 to -7). Plates were kept overnight at 37°C, after which colonies were counted with a maximum count of 380 Colony Forming Units (CFUs).

4.1.3. DNA extraction and qPCR

DNA extraction was performed on three different occasions. DNA was extracted from all SKC filter samples (DNA extraction protocol without enzymes) but only from 42 Pall filter samples (DNA extraction with the use of extra enzymes) – due to lack of enzymes – in two series. During the extraction of the first 25 Pall filter series, 8 samples were negatively compromised (partial DNA loss) and consequently DNA from a second series – containing 17 extra samples – was eventually extracted.

The first steps of the protocol were the same for all filter samples. Tubes containing the filters were added up to a volume of 5 ml with a solution consisting of Aqua B Braun water (Braun Melsungen AG, Germany) with a 0.05% v/v Tween®20 (Sigma-Aldrich, Chemie BV Zwijndrecht, The Netherlands) concentration. All tubes were manually turned every 5 minutes for 1h. Subsequently all samples were centrifuged at 1000 rcf for 15 minutes, after which, 1 ml of fluid was removed and frozen for possible future endotoxin analysis.

The protocol for the SKC filters then continued as follows:
- 4 ml of lysis buffer (NucliSENS® easyMAG®, BioMérieux Clinical Diagnostics, Marcy l'Etoile, France) was added to the Greiner tubes and all samples were again manually turned for 1h, after which centrifugation followed (2000 rcf for 15 minutes).
- The fluid was transferred to a clean 15 ml Greiner tube and the filters were discarded.

The protocol for the Pall filters continued by the usage of extra enzymes and incubation steps as previously described in Van Meurs (Van Meurs et al., 2013):
- 100µl lysostaphine from *Staphylococcus staphylolyticus* (1 mg/ml (1:40); Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands) was added, as was 20µl of lysozyme (50 mg/ml (1:200); Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands) and all tubes were 35 minutes incubated at 37°C on a shaking platform.
- 400µl of recombinant protease K was now added (20 mg/ml (1:10); PCR grade, Roche Diagnostics Nederland B.V, Almere, the Netherlands), after which tubes were incubated at 55°C (water bath) and 95°C (water bath) – each incubation step taking 10 minutes.
- The rest of the 'SKC filter protocol' was now implemented as mentioned above.

The new Greiner tubes were marked and 50µl of Silica magnetic beads (BioMérieux Clinical Diagnostics, Marcy l'Etoile, France), 5µl MRSA internal control – *pUC19* (Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands) – and an extra internal control of $7x10^4$ spores of *Bacillus thuringiensis* (the latter only to the Pall filters samples; 50µl of a 1:10 diluted spore suspension – Raven Labs, Omaha, Nebraska, USA) were added. After 10 minutes incubation and homogenizing, DNA was now extracted by means of the NucliSENS® easyMAG® principle (protocol and materials ordered from bioMérieux Clinical Diagnostics, Marcy l'Etoile, France) using a magnetic holder (DynaMag-50, Invitrogen Dynal AS, Oslo, Norway) as previously described. (Hogerwerf et al., 2012) The rest of the protocol is explained in detail in Annex V.

DNA extraction yielded approximately 50µl DNA per sample, of which 3 µl was used in the qPCR reaction at a 10 times diluted DNA concentration. As already mentioned, two internal controls were included in the DNA extraction process (e.g. *pUC19* and *Bacillus thuringiensis*), but eventually no control or inhibition testing was performed, due to time constraints. The

extraction process also included negative control samples (named: 'dilutions -6 and -7' SKC + Pall – Annex IVab). The qPCR protocol – explained in Part I of this report – was used. Samples were run in triplicate and NTCs were used on each plate. The SYBR Green assays are considered the leading assays, the probe based assay was merely used to include an extra check for specificity and accuracy.

4.1.4. Data evaluation

All results were analyzed by the Bio-rad CFX 384™ Manager software, Microsoft Office - Excel 2007 and 'R' statistics. Data was analyzed at a threshold of 40,5 Relative Fluorescence Units (RFUs).

4.1.5. Validation parameters

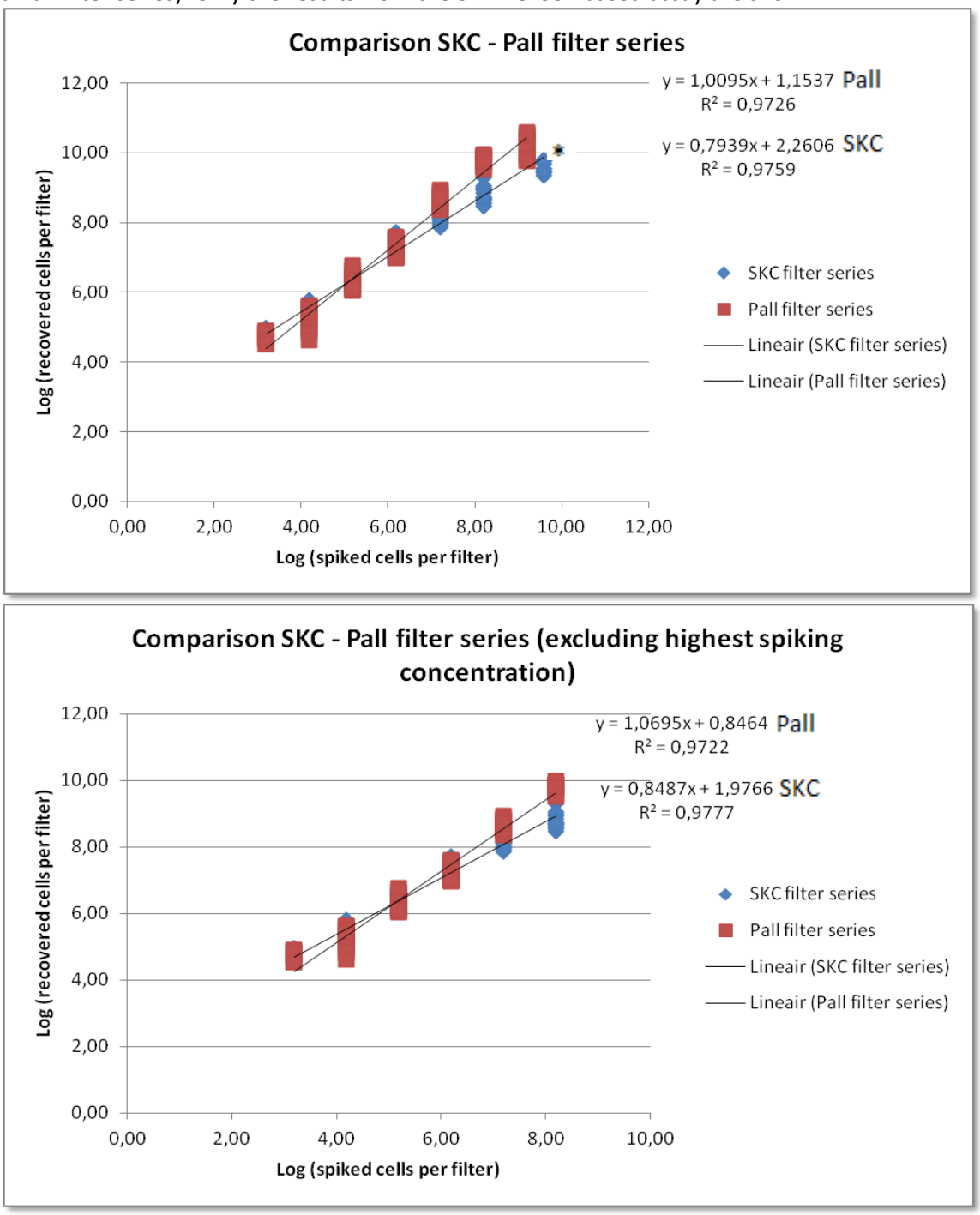
There are different ways to refer to the detection limit (LOD). It can be defined as the minimum single result which, with a stated probability, can be distinguished from a suitable blank value and the point where, with a stated probability, one can be confident that the signal due to the measurand can be distinguished from the instrumental background signal. (Burns and Valdivia, 2008) For a specific analytical procedure one can also refer to the lowest amount of an analyte in a sample, which can be detected but not necessarily quantified as an exact value. (Burns and Valdivia, 2008) In this study the LOD is specifically expressed as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time. (Burns and Valdivia, 2008) In detail, all technical replicates per sample with a C_q > 32,5 were considered negative as defined in Part I of this report. Samples were considered positive if at least two out of three technical replicates were positive. All positive samples were pooled per dilution and used in the calculation of the detection limit. Detected gene copies were corrected for qPCR volume, used qPCR dilution, elution volume and the amount of gene copies per cell (=1), and were expressed as the amount of recovered cells per filter. The counted CFUs on the 'control plates' were expressed as the amount of spiked cells per filter.

Certain validation parameters were used to validate the experiment i.a. precision, accuracy, sensitivity and specificity. Precision can be separated into total precision (reproducibility) and precision associated with the analytical instrument (instrument repeatability). (Hospodsky et al., 2010) Here, precision was measured by spiking multiple filters with the same bacterial concentration, extracting the DNA and calculating the coefficients of variation (CVs) between different sample replicates according to spiked concentration (instrument repeatability). In addition, Pall filters were extracted at different points in time and were compared. Also the assay executed with SYBR Green and the probe based assay were compared. Accuracy – the degree of closeness to the true value (Hospodsky et al., 2010) – of the experiment was monitored by comparing the recovered cells per filter with the spiked cells per filter. Analytical sensitivity was specified as the percentage of positive samples at a given dilution. Finally, specificity of the whole assay was measured by the percentage of positive samples among the negative control samples. (Travis et al., 2011)

4.2. Part II: Results

The amount of recovered cells per filter (log) after the spiking process, is presented in Figure IV.

Figure IV: Amount of recovered cells per filter by spiking concentration and spiking series (SKC filter and Pall filter series). Only the results from the SYBR Green based assay are shown.



Key: Upper graph: All spiking concentrations are shown. Lower graph: The highest spiking concentration is excluded. C_q cut-off = 32.5.

*This SKC spiking concentration was derived from a different spiking series (Series B – refer to Annex IVa+b) resulting in a different amount of spiked cells in comparison to the same spiking concentration from the Pall series. All other spiking concentrations were derived from the same spiking series (Series A). Points on the regression curve all represent positive qPCR replicates and not individual samples.

Table III: Results detection limit study: SYBR Green based assay.

Results per validation parameter	SKC filter series	Pall filter series
Sensitivity - Highest spiking dilution with positive samples - % sensitivity at the above spiking dilution	- 10^{-5} - 1560 spiked cells per filter - 33% - 2/6 positive samples - 2/6 samples with three positive replicates; 3/6 samples with one positive replicate (= 50% positive replicates) - 10^{-4}: 100% positive samples	- 10^{-5} - 1560 spiked cells per filter - 50% - 2/4 positive samples - 2/4 samples with 2 positive replicates, 2/4 samples with 1 positive replicate (= 50% positive replicates) - 10^{-4}: 100% positive samples
Specificity	- 100% - 0/11 positive samples - 0/33 positive replicates	- 88% - 1/8 positive samples - 1/24 positive replicates (96% specificity)
Variation/Precision - CVs calculated among recovered cells per filter (qPCR technical replicates per spiking concentration)	- 35,68 – 62,6	- 26,03 – 66,3
Accuracy - Events resulting in lower accuracy of the total assay (explained more in detail in 4.3.)	- More cells were recovered than spiked - 1 ml fluid removal during the DNA extraction process for future endotoxin analysis (also leads to loss of precision) - Specificity figures pointing to contamination - Laboratory-based assay - No inhibition testing - Different total numbers of bacteria per spiking concentration	- More cells were recovered than spiked - 1 ml fluid removal during the DNA extraction process for future endotoxin analysis (also leads to loss of precision) - Specificity figures pointing to contamination - Laboratory-based assay - No inhibition testing - Different total numbers of bacteria per spiking concentration
Calculated LOD (between brackets: calculations at the 95% confidence level) - Calculated among all positive samples per spiking concentration - Calculated among all positive technical replicates per spiking concentration	- 2182 (1549-15488) - 2181 (1549-15488)	- 2183 (1549-15488) - 2178 (1549-15488)

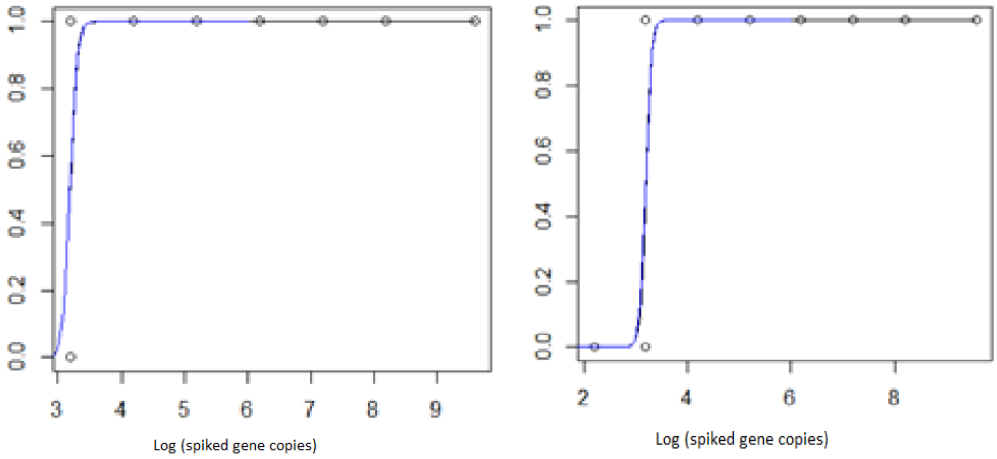
The results of the detection limit study are presented in Table III. The regression curve (Figure IV) is nearly linear from 10 times the original DNA concentration towards a 10^{-5} spiking concentration. What stands out first when examining the results more closely, is that more cells per filter were recovered than were applied to the filters, especially in the Pall filter series. This discrepancy also seems to increase towards a higher amount of spiked cells and was confirmed by the probe based assay (figures not shown). Furthermore, when comparing the two different spiking series, the regression coefficient of the Pall series lies closer to one and the corresponding curve is steeper, resulting from higher yields at higher spiked bacterial concentrations.

The coefficients of variation (CVs) of the SKC and Pall filter series, calculated among recovered cells per filter, are respectively located between 35,7 – 62,6 and 26,03 – 66,3. No

big differences were observed between the two extraction series of the Pall filter assay, except when one considers the highest spiking concentration. At the highest spiking concentration, the mean amount of cells recovered by Pall series 1 (= t1) equals 1,17E+09 recovered cells per filter versus 1,71E+08 recovered cells by Pall series 2 (= t2). Remarkably, the noted variation among the replicates per spiking concentration, is apparently greater than the difference between 'compromised' and not 'compromised' Pall filter samples. This means that no real difference could be noted between these samples, with the exception of one sample. The latter sample presented a very low amount of recovered cells per filter in comparison with the rest. This fact is considered remarkable, because fluid – including magnetic beads –, was lost from the other (7) compromised samples, when performing the NucliSENS® easyMAG protocol (Annex V: step 7-9).

The detection limit – based on 95% probability of possible detection – cannot be expressed in one number. The LOD of the SKC filter and Pall filter assay was respectively calculated as 2182 and 2183 absolute gene copies (= separate *mecA* containing bacterial cells), when using the SYBR Green based qPCR assay. When using a probe based assay, these figures were respectively 2194 (SKC filter assay) and 1955 (Pall filter assay). However, for all these calculations a general linear model (glm) was used and a fitting probability of nearly one at all calculations was observed. Therefore, the above figures could not be determined at the 95% confidence level. However, it can be estimated with at least 95% certainty, that these figures will lie somewhere between 1548 and 15488 CFUs per filter (= 3.19-4.19 log (gene copies)) based on the glm curves (an example is shown in figure V). At the lowest spiking concentration (10^{-5}), not all samples were positive, but also not all were negative. If assuming that at a lower spiking concentration (e.g. 10^{-6}) all samples are negative, the calculated detection limit is only marginally lowered for all assays. The figures from the SYBR Green assays and the SKC filter/probe based assay, are reduced by 22-25 CFUs. The figure from the Pall filter and probe based assay is reduced by approximately 61 CFUs. Yet, also these figures could not be determined with 95% certainty.

Figure V: An example of the outcome of the used general linear model – SKC, SYBR Green assay, percentage of detection by spiking concentration (log).



Key: Y-axis: qPCR detection expressed as zero (not detected) or 1 (detected). If multiplied by 100 = the percentage of detection at a given spiking concentration is presented.
Left graph = Original graph
Right graph = Extra spiking level (2.19 = 10^{-6}) is added of which all samples are negative.

As mentioned in Table III, SYBR Green based SKC filter assay specificity is 100% and the Pall filter assay specificity is 88%. All assays (SKC + Pall) with probe, showed a 100% specificity.

4.3. Part II: Discussion

To the best of the author's knowledge, only one good qPCR air filter detection limit study can be found in the literature. (Hospodsky et al., 2010) This study describes an estimation of accuracy, precision and LODs associated with qPCR of air sampling filters loaded with *Aspergillus fumigatus*, *Bacillus atrophaeus* and *E. coli*. However, the detection limit (*A. fumigatus* and *B. atrophaeus*) was calculated in a different manner, using serial spiking dilutions of standard DNA samples, corresponding to know cell numbers and by identifying the minimum cell concentration where seven of seven (99% confidence level) qPCR analyses where positive (Figure VI). The outcome of this calculation (number of cells) was divided by the %DNA extraction efficiency (η_{DNA}) and the %extraction efficiency of whole cells and spores from sampling filters (η_{filter}). This result was multiplied by the ratio of total extracted DNA to DNA used as template in the qPCRs. η_{DNA} was calculated as the DNA mass extracted from microorganisms in aqueous solutions (estimated with a PicoGreen assay) divided by an estimated theoretical mass of DNA per cell for the specific microorganism, multiplied by 100. η_{filter} was calculated by the recovered DNA mass (in pg) from a known quantity of spiked cells onto clean and PM-loaded quartz fiber or PCTE filters, divided by the recovered mass of DNA (in pg) from the same number of cells originally spiked into PBS, multiplied by 100.

Figure VI: Calculations MDL, η_{DNA} and η_{filter} as described in Hospodsky et al., 2010

$$\text{Method Detection Limit (MDL)} = \frac{\text{qPCR instrument MDL}}{(\eta_{DNA})\,(\eta_{filter})}$$

$$\eta_{DNA} = 100 \times \frac{\text{Measured DNA mass recovered}}{\text{Theoretical DNA mass}} \qquad \eta_{filter} = 100 \times \frac{\text{Measured DNA mass recovered from filter}}{\text{Measured DNA recovered from aqueous solution}}$$

Although partly a different method was used in this study to estimate the detection limit, some points in the study can be used to compare the results of the current study and will be described throughout the following.

In the current study, at the C_q cut-off, not all samples were positive at the 10^{-5} spiking concentration, representing 1560 gene copies and a sensitivity of 33% (SKC + Pall SYBR Green based assays). However, all samples were still located in the linear range. Also only fairly limited variation between samples of the same dilution was seen, as compared to the results of 'Hospodsky et al.'. In the latter study, at the concentration of 10^3 spiked cells, the calculated instrument repeatability for *E. coli* was 36% and 11% (resp. Quartz, PCTE filters) and 57% and 58% for *B. atrophaeus* (resp. Quartz, PCTE filters). At the concentration of 10^4 spiked cells, the percentages were 44% and 26% for *E. coli* (resp. 'Quartz' and 'PCTE') and 41 and 51% (resp. 'Quartz' and 'PCTE') for *B. atrophaeus*. Figures from the current study at 10^{-4} (±15000 cells) and 10^{-5} (± 1500 cells) spiked cells, instrument repeatability were respectively situated around 50% and around 58%. However, it should be noted that the instrument repeatability's from the cited study, were calculated among seven replicates per spiking

concentration, which is higher than the amount of replicates used in this study (SKC: 6 replicates at both spiking concentrations; Pall: 10^{-4}, 5 replicates – 10^{-5}, 4 replicates).

The detection limit couldn't be defined with 95% certainty and is situated somewhere between 1549 and 15488 spiked cells per filter for all assays. In the aforementioned detection limit study on aerosol filter samples, a detection limit of 2-3 cells (*B. atrophaeus*) was found, which is much lower than the detection limit found in the current study. Yet, the figures from the current study at the 10^{-5} spiking concentration are all situated around the LOQ of the qPCR and correspond fairly well to the theoretically calculated amount of copies per qPCR as presented in Annex IVa+b (±factor 10 difference).

The most remarkable observation is probably that more cells were recovered than spiked at nearly all spiking concentrations. This shows that during the extraction process, not much DNA has been lost. Yet, this observation leads to low assay accuracy, as of course such observation cannot be completely true. Therefore, no reliable recovery rates could be calculated. Three possible explanations for this inaccuracy can be thought of. Firstly, the concentration of extracted DNA used for the generation of the qPCR calibration curve to calculate gene copies, could be overestimated. As already explained in Part I of this report, a new calibration curve was designed from recovered *mecA* qPCR product, which pointed to the initial overestimation of the extracted DNA. The purified qPCR product DNA – used for the generation of the new calibration curve – showed a more typical spectral image when analyzed by NanoDrop, in combination with improved absorbance ratios, in comparison to the DNA used for the old calibration curves. Yet, this still does not 100% guarantee a good estimation of the DNA concentration, as previously described in the literature. (Simbolo et al., 2013) Alternatively, the high recovery to spiking rate can also be the result of an underestimation of the counted colonies on the control plates, due to large amounts of death bacteria. qPCR does not differentiate between death or viable bacteria. The presence of multiple death bacteria in the spiking fluid during the spiking process, could theoretically result in a reduced amount of bacterial colonies on the 'control plates' used for colony counting. During bacterial development, bacteria experience several phases, starting with the lag phase and subsequently go through an exponential and stationary phase, ending in bacterial death. (Quinn et al., 2002) When bacteria are spiked too late (e.g. at or after the stationary phase = between 10-24 hrs after inoculation (Luppens et al., 2002)) – in this process, fewer bacteria will survive the spiking process and form colonies on the control plates. The exact interval between the setting up of the culture and the spiking of the filters was around 20-23hrs, which in theory could already result in bacterial death. A third possible explanation for the finding of more recovered cells per filter than spiked, is the possible overestimation of the qPCR generated figures, due to low specificity of the qPCR. As earlier noted, the assay with probe should be more specific than the SYBR Green based assay. Comparing the results from the probe based assay – figures not shown – with the assay without probe, a less pronounced difference is seen between spiked and recovered cells per filter. Yet, the difference is still present throughout both SKC and Pall filter assays.

As mentioned in the DNA extraction protocol, during the extraction process, a total of 1 ml fluid was collected (from the 5 ml fluid containing the spiked bacteria) for future endotoxin analysis, after initial centrifugation. The time of fluid removal can be debated, but probably results in a decrease of the final amount of extracted DNA. In the end, a correction factor of

0,8 (20% loss) was used, however, this can possibly overestimate the DNA loss when the bacterial cells are not evenly distributed throughout the sample. Because of this, the event not only contributes to loss of precision, but also to loss of accuracy.
Another notable finding is the deflection of the SKC filter series regression curve, as seen in Figure IV at higher spiking concentrations, in comparison with the Pall filter series curve (confirmed by the probe based assay). Moreover, as the regression coefficient of the Pall filter series lies closer to one – also when the highest spiking concentration is excluded (Figure IV bottom graph) – this could be an indication that the extraction protocol from the Pall filter series, results in higher DNA yields at higher spiked bacterial concentrations. The DNA extraction protocol, used for the Pall filter series, uses more enzymes and incubation steps – as described in 4.1.3. – in comparison with the 'SKC filter DNA extraction protocol'. This can possibly account for the observed difference in recovered cell counts between the SKC and Pall filter assay. Dissimilarities resulting from the use of different DNA extraction protocols, are in fact not surprising, since larger differences are known to exist between this kind of protocols. (Pontiroli et al., 2011)

The assay specificity of the SKC filter assay turned out to be 100% (C_q cut-off 32.5). However, one Pall filter sample (1/3 qPCR replicates) was found to be positive (specificity Pall filter assay 88%) at a $C_q < 32,5$. This could be due to contamination of the samples during spiking, the DNA extraction process or the qPCR reaction itself. Also it could be the result of non-specificity of the primer pair, as explained in Part I of this report. As the assay with the more specific probe also confirmed the presence of amplified product in the negative controls (however only at C_qs > C_q cut-off) – figures not shown –, this contamination more likely occurred during the DNA extraction process. Contamination during spiking is also somewhat less likely, as the monitoring of sterility during this process is more easily accomplished than compared to the monitoring during the extraction process. For instance, the Pall filter extraction protocol includes multiple enzyme incubation steps and even the use of water baths. This increases the risk of contamination considerably by e.g. aerosol formation and the spreading of sporadically occurring bacteria or DNA on the outside of the tubes (4.1.3.). At this moment, this and other possible sources of contamination are still being investigated, however, raising some concerns on the reliability of the current assay.

In addition to the above results, some other remarks need to be added. Firstly, for this particular study inhibition has not been tested yet. qPCR reactions were only run with 10-times diluted DNA. When sampling air, it is well known that even modest amounts of particulate matter (≤ 50µg) from dust and microorganisms in air can cause complete or partial inhibition of PCR reactions (McDevitt et al., 2007), necessitating the use of internal controls to check for inhibition. During this study no real air sampling was performed, limiting the accuracy of the assay, but also limiting inhibition. Yet, extra bacteria (i.a. *E. coli* spp.) were added, possibly influencing the outcome of the test results. The inclusion of internal controls (*pUC19* – SKC and Pall filter assay, *Bacillus thuringiensis* – only Pall filter assay) makes future inhibition testing nevertheless possible. Another drawback to the study design is the mutually varying total cell count spiked on the filters. Mainly spiking concentrations '+1 (10^7)', '0 (10^6)' and '-1 (10^5)' (Annex IVa+b) differ substantially from the other spiked concentrations (10^4), when looking at the total amount of spiked bacteria. This fact ensures a somewhat less reliable direct comparison between concentration steps. Another point of remark concerns the spiking technique. The used spiking technique of the

filters inside a Greiner tube, didn't prove to be quite successful. I.a. the specific water repellent characteristic of the filters, in combination with the tapered end tubes containing the filters, prevented sufficient contact time between the spiked bacteria and the filter surface. However, during the extraction phase, all filters were totally immersed in a mixture of Aqua B Braun water and Tween 20, at least permitting some contact time between the filter and the spiked fluid, as was the case for the rest of the extraction process. For future testing it is however recommended to develop a more appropriate spiking technique e.g. the use of petri dishes instead of Greiner tubes or more complicated by using sterile filtration galleries as explained in Hospodsky et al., 2010.

4.4. Part II: Conclusion

In conclusion, for this particular study, the detection limit is situated somewhere between 1549-15488 cells per filter. At the 10^{-5} dilution, the analytical sensitivity ranged from 33%-50%, however no spiking dilution with 0% positive samples was included in the study design. When the logs of the amount of cells spiked on the filters are plotted against the number of recovered cells per filter, the resulting regression curve is very linear from spiking concentration +1 until -5 and the recovered gene copies per qPCR reaction correspond rather well with the initially predicted amount of gene copies per qPCR reaction. At high bacterial concentrations, the Pall filter assay is considered more appropriate, when used in combination with the 'DNA extraction protocol with enzymes'. The assay precision is considered fairly modest, but could have been influenced by the removal of fluid during the extraction process for endotoxin analysis. In addition, a lot of other shortcomings to the study can be noted. First of all, the specificity of the Pall filter assay was only 88%, indicating possible contamination during the DNA extraction process. Also, the assay's accuracy can be greatly questioned. Not only is this study a laboratory-based assay and consequently no true cell filter interactions produced by impaction and interception can be measured when sampling air (Hospodsky et al., 2010), also no influence of real particulate matter was investigated. In addition, more cells were recovered from the filters, than spiked, which however shows that the DNA extraction process didn't result in great DNA losses. Also, no inhibition testing was included in this study. Furthermore, the spiking technique itself should be redesigned for future detection limit testing. It should be noted however, that to the best of the authors knowledge, only one good article is published on LOD testing of air filter samples and this project is consequently considered a good first attempt to investigate this matter.

5. Part III: Detection of *mecA* in manure, soil and air

5.1. Part III: Material and Methods

As mentioned before, for a comprehensive description of the SKBII materials and methods used – beyond what has already been described in the introduction to this report –, the reader is referred to: (Schmitt et al., 2013). The *mecA* qPCR was run according to the protocol designed in part I of this report. All DNA samples (3µl per sample) – derived from manure, soil and air filters – were initially analyzed with the primer pair *François*, with and without the corresponding probe (initial DNA dilutions: manure 10x, soil 10x and 500x, air filters 10x). As a consequence of the low amount of positive technical replicates found during these runs (see below), the Most Probable Number (MPN) method was considered and partly applied.

5.1.1. Data evaluation

All results were analyzed by the Bio-rad CFX 384™ Manager software, Microsoft Office - Excel 2007 and 'R' statistics. Data were analyzed at a threshold of 40,5 RFUs.

5.1.2. Most probable number (MPN) - qPCR

The Most Probable Number (MPN) method is based on the assumption that one bacterial cell will induce growth in a single tube containing liquid growth medium. The essence of this method is – as described in the Bacteriological Analytical Manual (BAM) of the U.S. FDA (Blodgett, 2010) – to dilute (e.g. decimal series) the original sample containing the micro-organism of choice to such a degree that the inoculated tubes will sometimes but not always contain viable organisms. By selecting all replicates from three specifically chosen serial (e.g. decimal) dilutions, the MPN – the original concentration of the target bacterium in the original sample – can be estimated by using a special spreadsheet or table (MPN statistics). MPN-PCR combines the principle of MPN with the use of the PCR technique. (Sharma et al., 2007) The MPN method can be combined with conventional PCR, however also the combination with qPCR has been used (MPN-real-time PCR), mostly for reasons of speed and simplicity in comparison to the MPN method alone. (De Martinis et al., 2007, Aparecida de Oliveira et al., 2010) Also soil samples have already been analyzed this way. (Schulz et al., 2010) Extracted template DNA or RNA is diluted multiple times (end point dilution) and the original target concentration in the original template is estimated as the most probable number by the different (q)PCR dilutions replicates. The use of the Poisson distribution is justified under the assumption that genetic material is randomly distributed within the sample and that a single copy of the target provides a positive signal. (Sharma et al., 2007, Rutjes et al., 2005, Blodgett, 2010) The amount of PCR detectable units (PDU) in the original template can subsequently be calculated by relating this figure to the investigated volume. (Rutjes, 2004) Spreadsheets and special MPN calculators to assist in these calculations are available on the internet. (e.g. U.S. Food and Drug Administration (FDA), dr. R. Blodgett - http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm - bottom of the internet page; EPA – USA Environmental Protection Agency – MPN Software Program: http://www.epa.gov/microbes/online.html - bottom of the internet page. (Varughese et al., 2007)

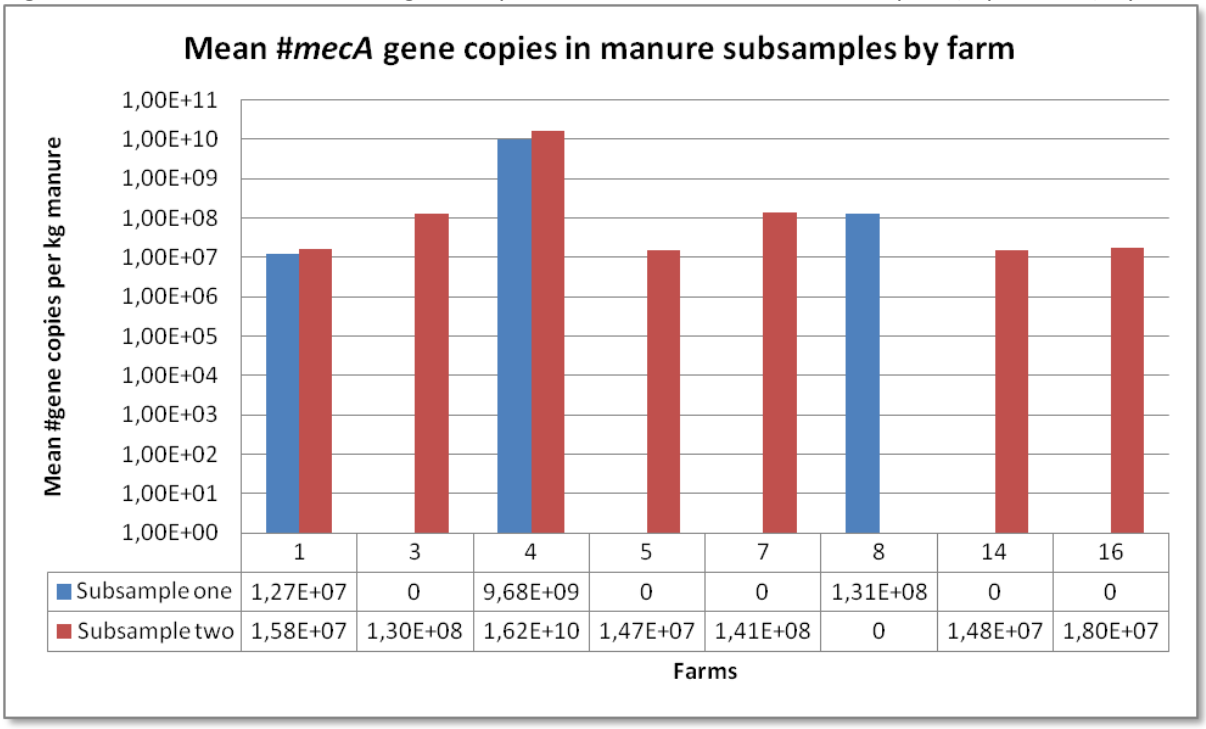
To assess the initial amount of DNA in soil, manure and air samples, MPN-qPCR was considered, due to lack of enough positive technical replicates, after initially running these samples by qPCR. As such, different DNA concentrations were evaluated in an 'MPN-qPCR' (soil samples: 3 replicates of the original DNA, 8 replicates of 10x diluted and 3 replicates of 100x diluted DNA). Air filter samples were also analyzed at the original (2 technical replicates) and 10 times diluted DNA (8 replicates) concentration.

5.2. Part III: Results

5.2.1. Results manure samples

The results of the *mecA* qPCR demonstrated only low amounts of gene copies in the examined manure, soil and air filter samples. At a C_q cut-off of 32.5 (LOQ = 86 gene copies in 3µl DNA template), all 16 qPCR replicates of the manure subsamples (two per farm) belonging to farm 4, were found positive (mean = $1{,}29 \times 10^{10}$ gene copies per kg manure) (Figure VII). At the C_q cut-off, at least two other positive qPCR replicates in reference to a single manure subsample were found in manure samples from farms 1, 3 and 5. From the manure (sub)samples corresponding to the farms 7, 8, 14 and 16 only one technical replicate was positive. Gene copies numbers per kg manure were all situated between 10^7-10^{10}. If the C_q cut-off was not respected, more replicates from qPCR manure samples per farm and more farms in general were found positive (Annex VI). These C_qs mostly lie between 32.5 and 35.

Figure VII: Mean absolute *mecA* gene copies detected in manure subsamples (2 per farm) by farm.



	1	3	4	5	7	8	14	16
Subsample one	1,27E+07	0	9,68E+09	0	0	1,31E+08	0	0
Subsample two	1,58E+07	1,30E+08	1,62E+10	1,47E+07	1,41E+08	0	1,48E+07	1,80E+07

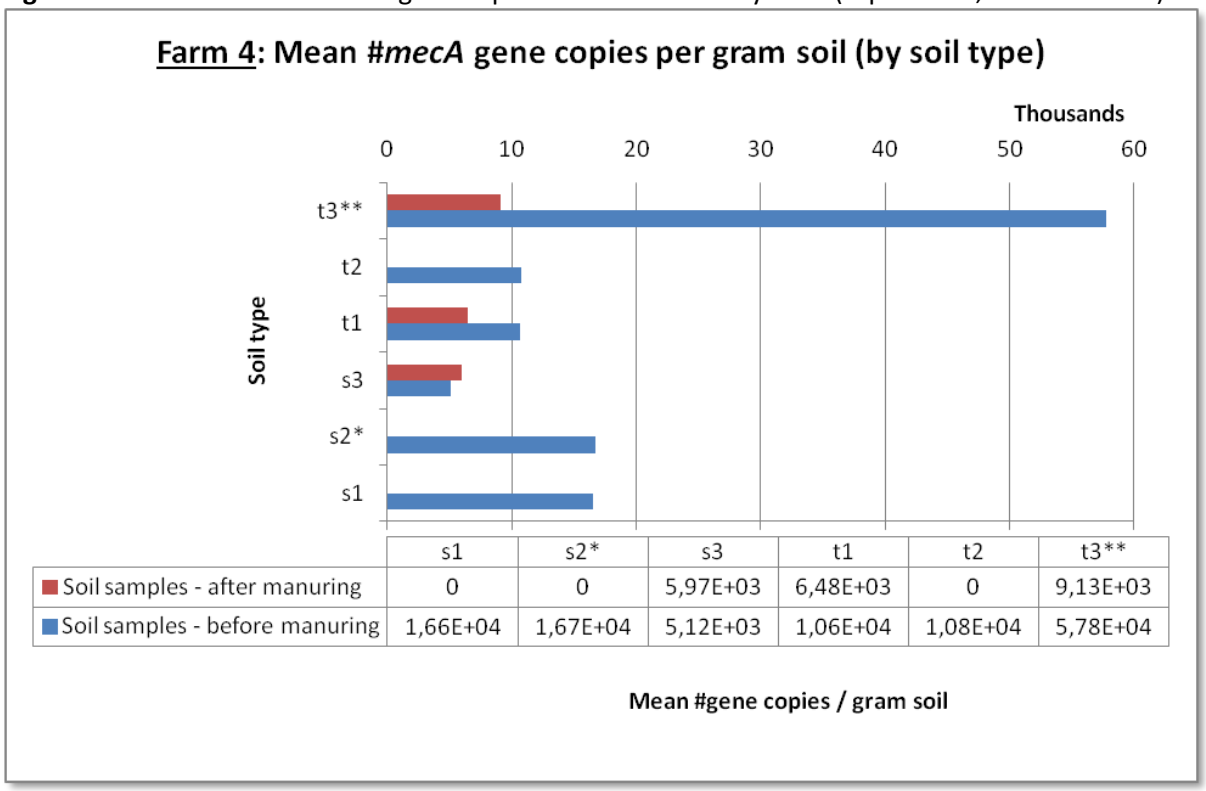
Key: 'Zeros' represent a gene copy number lower than the LOQ or an amount of gene copies lower than the detection limit of the mecA qPCR.
Kind of manure by farm: 1: pigs, 3: poultry, 4: poultry + pigs, 5: pigs, 7: pigs, 8: pigs, 14: pigs, 16: pigs.

5.2.2. Results soil samples

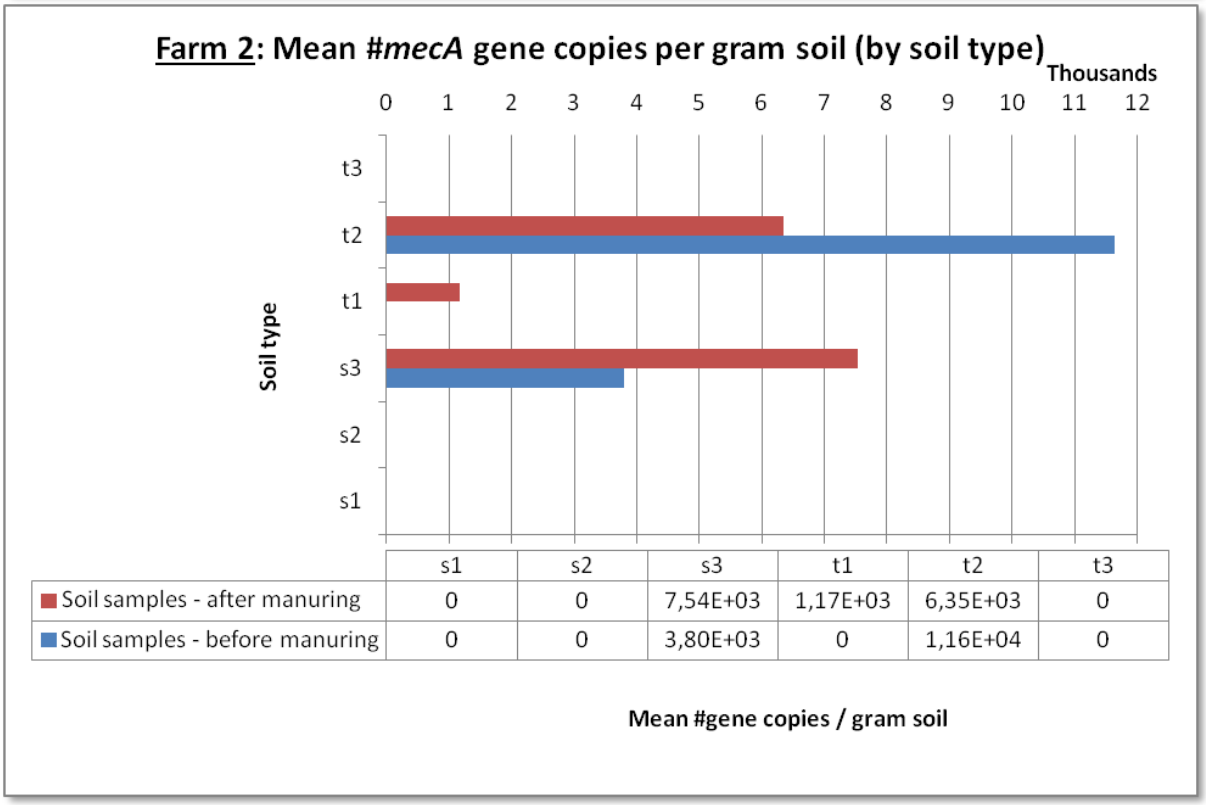
Per farm two different soil types (topsoil and 'deep layer soil') from three different locations in the field (= six mixed samples derived from subplot samples) were analyzed. No soil samples were found positive at the C_q cut-off. The results of the initial qPCR showed that most samples with C_qs larger than the C_q cut-off, were only positive in less than two out of three technical replicates. Subsequently, samples from farms with at least two positive replicates were selected and rerun at the MPN-qPCR DNA concentrations. The MPN-qPCR also didn't provide positive samples above the LOQ. Nevertheless, at least two technical replicates (/8) from two different soil types corresponding to farm 4 were found positive (farm 4: total of 9 'positive' samples > C_q cut-off). In total (not including farm 4) 8 different samples from 5 farms showed a positive C_q (representing 10^3-10^4 gene copies/gram soil). However of these, none showed more than one positive technical replicate (/8 replicates).

Following the evaluation of the manure samples, it was calculated that manure from farm 4 likely could not be detected in soil after the manuring process, as the expected transferred amount of gene copies (~34-37 genes per qPCR reaction) lied below the expected detection limit of the qPCR. To a larger extent this also applies to the other farms with positive soil samples. Samples from two farms with at least two positive samples (however C_qs > C_q cut-off) are summarized in Figure VIII.

Figure VIII: Mean absolute *mecA* gene copies detected in soil by farm (top: farm 4, below: farm 2).



	s1	s2*	s3	t1	t2	t3**
Soil samples - after manuring	0	0	5,97E+03	6,48E+03	0	9,13E+03
Soil samples - before manuring	1,66E+04	1,67E+04	5,12E+03	1,06E+04	1,08E+04	5,78E+04



	s1	s2	s3	t1	t2	t3
Soil samples - after manuring	0	0	7,54E+03	1,17E+03	6,35E+03	0
Soil samples - before manuring	0	0	3,80E+03	0	1,16E+04	0

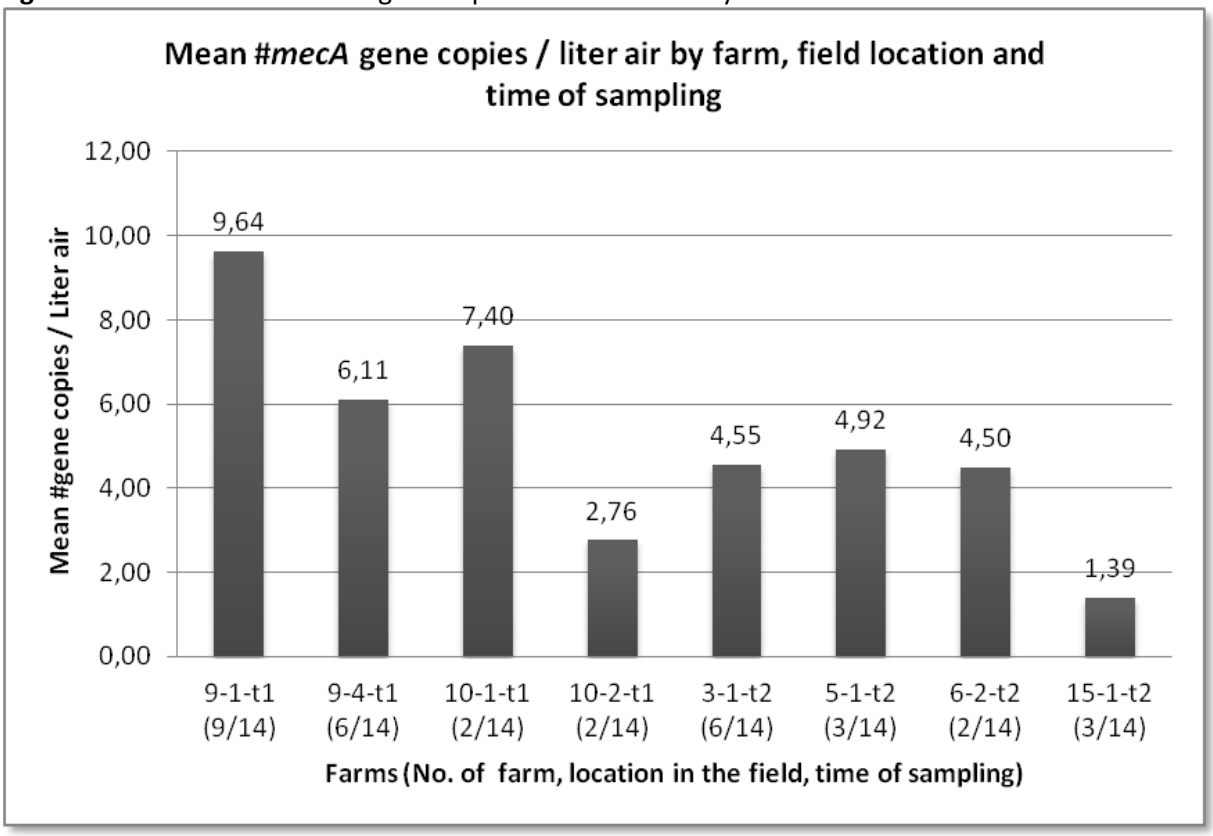
Key: Soil types: s1-3 = deep layer soil, subsample 1-3; t1-3 = topsoil, subsample 1-3. * = Data 'before manuring' based on 2/8 replicates (10x diluted) ** = Data 'before manuring' based on 5/8 replicates (10x diluted) and 1/3 (100x diluted) replicates. Rest of the data on gene copies is based on 1/8 replicates (10x diluted).

5.2.3. Results air filter samples

As with the soil samples, after the initial qPCR run, all air filter samples (minimum of 4 per farm) with at least two positive replicates were selected and rerun. Only two samples had C_q values ≤ C_q cut-off. Of these, one sample of farm 9 (upwind the field before manuring) showed two positive replicates (2/2 replicates at the original DNA concentration). At C_q values > C_q cut-off more replicates were positive (Figure IX).

Per farm at least one negative control filter (blanco filters) was included in this study. The negative controls from farms 4 (1/3 replicates), 9 (1/3 replicates), 10 (t1: 1/3 replicates, t2: 8/14 positive replicates), 14 (1/3 replicates) and 15 (1/3 replicates) were found positive, although at a C_q ≥ the cut-off.

Figure IX: Mean absolute *mecA* gene copies detected in air by farm.



Key: First No. = farm No., second No. = location in the field accoring to the gradient, third number = time of sampling (before = t1 and after = t2 manuring), in between brackets = amount of positive replicates with no respect to the C_q cut-off (14 replicates = 3x original DNA, 8x 10 times diluted DNA, 3x 100 times diluted DNA).
Gradient: 1. Upwind the field, 2. 5m within the field upwind, 3. Middle of the field, 4. 5m within the field downwind
Sample 9-1-t1: The absolute gene copy number per liter air solely based on the C_qs ≤ 32,5 = 7,6.

5.3. Part III: Discussion

With respect to the C_q cut-off of 32.5, manure samples from farms 1, 3, 5, 7, 8, 14 and 16 were found positive and only the amount of qPCR replicates from farm 4 indicate a strong presence of *mecA* gene copies (mean = $1{,}29 \times 10^{10}$ / kg manure). Manure from farm 11 could not be tested due to lack of original DNA. With the exception of manure samples from farm 4, no reliable quantitative statements can be drawn from the gene copies in manure when

considering the limited number of positive technical replicates with C_qs below the cut-off. The same applies to the soil and air filter samples, as only one filter sample (farm 1) positively stands out.

The main goal of Part III of this research project was to compare the *mecA* qPCR data with the 'MRSA ST398 qPCR' generated figures. In general, the data from the *mecA* qPCR confirms the lack of MRSA (presented by *mecA* gene copies) detected in soil and air. The manure samples however, differ greatly from the data generated with the ST398 qPCR (Table IV). Farm 4 is clearly positive in the *mecA* qPCR, while being negative in the ST398 assay. This could be explained by the presence of non-MRSA ST398 spp. or even the presence of *mecA* carrying CNS in this farm. These latter staphylococcal spp. have previously been identified in Dutch farms. (Tulinski et al., 2012) As the farmer used pig and poultry manure for fertilizing, no clear origin of the *mecA* genes can be designated. Unfortunately, also no details on possible antibiotic use were provided by this farmer. Manure (pigs) from farm 1 contained a mean amount of 1,42 $x10^7$ *mecA* gene copies per kg manure, which confirms the finding of ST398 gene copies. The presence of these *mecA* gene copies are therefore probably solely due to the presence of MRSA ST398 in manure from farm 1. The other 'positive farms' (farms 7 and 14) by the ST398 qPCR couldn't be confirmed by the *mecA* qPCR by reliable gene copy number estimations. However, if less qPCR technical replicates per sample and higher C_qs would be allowed, except for farm 2, the estimations of the total amount of *mecA* gene copies correspond fairly well to the findings of the MRSA ST398 assay. Also the amount of qPCR replicates were more or less in line among the two assays. Nevertheless, differences are seen between the outcomes of the two assays. This can be expected as the amount of gene copies in most of these samples are situated around or below the expected limit of detection of both qPCRs.

The in general low positivity of these manure samples among farms (ST398: 4 farms (30% of pig farms), *mecA*: 2 farms (15% pig farms)) – predominantly coming from pigs (Annex VI) – differs from the high MRSA prevalence among Dutch pig farms as mentioned in the introduction to this report. (Wagenaar and Van De Giessen, 2009) As qPCR techniques are less suitable for demonstrating lower levels of bacterial DNA, a higher overall prevalence cannot be ruled out. In the above mentioned study i.a. pigs (pooled nose swabs) and dust from the farm was sampled. MRSA was cultured using two consecutive steps of selective enrichment before plating on a MRSA selective plate. MRSA presence was confirmed by the use of a multiplex PCR and isolates were *spa* typed. Also antibiotic susceptibility testing was executed. The use of enrichment techniques, as practiced in the aforementioned study, is known to increase the bacterial screening sensitivity. (Brown et al., 2005, Dierikx et al., 2013) Also the fact that a farm was considered MRSA positive if only one dust or one pooled swab sample was positive, has probably contributed to the large farm prevalence. As already mentioned in the SKBII report, the low prevalence in this study could also be the result of the selection of the 'best pupils in the class', since farmers intentionally and voluntarily participated in this research project and are therefore probably more engaging at lowering their farm disease status (selection bias).

Table IV: Comparison of the qPCR results of the manure samples by farm: ST398 vs. *mecA* qPCR.

Farms	# Positive qPCR replicates/ total replicates (ST398)	#Gene copies / kg manure (ST398)	# Positive qPCR replicates / total replicates - only replicates with C_qs ≤ 32,5 (*mecA*)	# Positive qPCR replicates / total replicates - including replicates with C_qs > 32,5 (*mecA*)	#Gene copies / kg manure (only from replicates with C_qs ≤ 32,5) (*mecA*)
1	**7/12**	**2,19E+07**	**3/6** + 0/16 + 0/6	6/6 + 5/16 + 0/6	**1,42E+07** (mean from two subsamples)
2	**9/12**	**2,02E+07**			
3			0/6 + 3/16 + 0/6	0/6 + 9/16 + 1/6	1,30E+08
4			**6/6** + **16/16** + 0/6	6/6 + 16/16 + 2/6	**1,29E+10** (mean from two subsamples)
5	2/12		2/6 + 0/16 + 0/6	4/6 + 1/16 + 0/6	1,47E+07
6	1/12			0/6 + 1/16 + 0/6	
7	**10/12**	**7,01E+07**	0/6 + 1/16 + 0/6	4/6 + 4/16 + 0/6	1,41E+08
8	2/12		0/6 + 1/16 + 0/6	1/6 + 1/16 + 0/6	1,31E+08
9	1/12				
10	1/12				
11			NA	NA	NA
12	3/12				
13	1/12			6/6 + 6/16 + 0/6	
14	**6/12**	**2,47E+07**	1/6 + 0/16 + 0/6	2/6 + 2/16 + 1/6	1,48E+07
15				0/6 + 3/16 + 1/6	
16	2/12		1/6 + 0/6 + 0/6	5/6 + 3/16 + 1/6	1,80E+07

All data marked in grey is considered a reliable estimation of the amount of gene copies / kg manure based on the amount of qPCR replicates **and** a C_q ≤ C_q cut-off.

Key:
Tested concentrations *mecA* (amount of replicates per sample for 2 subsamples per farm): 3x - original DNA concentration (1st figure: 6/6), 8x - 10x diluted concentration (2nd figure: 16/16), 3x - 100x diluted concentration (3rd figure: 6/6).
Empty boxes represent zero positive samples.

It was calculated that manure from the investigated farms could not be traced back in soil after the manuring process, including soil samples from farm 4. Nevertheless, nearly all soil subsamples from farm 4 gave an indication of possible *mecA* presence in soil before and after the manuring process, with the signal being strongest (judged as most positive technical replicates, topsoil 3) after the manuring process. The strict LOQ in combination with the low amount of technical qPCR replicates among biological replicates, do not allow reliable estimations of the amount of *mecA* gene copies in soil. This confirms the lack of gene copies in soil by the ST398 qPCR (only 4 of 192 soil samples were positive). In addition, no relation with the manuring process can be detected. However the positive soil samples from farm 4 could possibly indicate the ongoing presence of *mecA* genes in (top)soil. This is partially the same for farm 2, where also some positive soil samples before and after the manuring process were detected.

By the ST398 qPCR assay, only two filters from farms 8 and 10 were found positive (4 and 4,6 gene copies per liter air). The *mecA* qPCR detected one positive filter from farm 1 (upwind position, mean = 7.6 gene copies) within the LOQ range (2/2 replicates). More filters were found positive if the LOQ was lowered, but as a lot of observations have high C_qs (+35) and because in general only one technical replicate was positive, the reliability of these gene copy estimations is strongly questioned. As such, no satisfying conclusions could be drawn from these observations, except for the fact that only a very limited amount of gene copies were found in air filters by the ST398 and *mecA* qPCR. As already mentioned in the SKBII report, these low amount of positive samples, could be the result of sampling in a wet period and the short time frame (~6 hours) in which air sampling took place. As a result of some organizational difficulties not all farms were sampled before and after manuring and possible transmission from soil to air could have been missed. It should be noted that a lot of negative control filter samples were found positive (6 samples from 5 different farms), however presenting C_qs below the C_q cut-off (mainly C_qs > 34) and a very low amount of positive qPCR replicates except for one sample. This fact emphasizes the importance of the C_q cut-off to distinguish between simple contamination and real positivity of the samples. As multiple sources could be the origin of this 'contamination', attempts are being made at this moment to investigate these possible causes.

Not a lot of studies have been carried out on the presence of MRSA in soil and air samples, especially in the Netherlands. For instance, a study among 27 German pig farms mentioned an inside pig farm MRSA prevalence of 85.2%, based on air sampling (predominately MRSA ST398). (Friese et al., 2012) In another German study (Schulz et al., 2012), LA-MRSA was isolated from ambient air and soil surfaces in the vicinity of pig farms of which all harbored LA-MRSA positive pigs. The authors describe a direct airborne transmission of LA-MRSA from the inside to the outside of the farm influenced by the wind direction. Sampling downwind air yielded 21% positive samples (5/24) at 50 and 150 meter, however distributed over four sampling seasons and at two distances from the farms (= 5/47). Also downwind accumulation on soil surfaces of LA-MRSA was assumed (distances 50, 150 and 300m yielded 73% positive samples compared to 33% positive upwind samples). Airborne transmission was likely influenced by season, meaning that significantly more positive soil and air samples were found in the summer. Both German studies used a duplex *nuc* and *mecA* qPCR to confirm MRSA presence in air and soil after culturing. (Pasanen et al., 2010) Especially the

figures from the latter study indicate that the occurrence of MRSA in air and soil is associated with pig farms, but also that even in the vicinity of a pig farm, it has been proven difficult to demonstrate MRSA in air samples, also in comparison with MRSA in soil surface samples.

Additionally, a Dutch study (Heederik, 2011) demonstrated that the concentration of *mecA* in particulate matter (PM10) was associated with the amount of farms in a radius of 1000m around the sampling point, as well as the detection of MRSA ST398 in PM10. Not only *mecA* was clearly more detected than ST398, also the concentration of *mecA* and ST398 correlated significantly in the measurements. Gradient measurements around farms also revealed higher concentrations of *mecA* inside a pig and poultry farm in comparison with outside upwind and downwind locations. *MecA* concentration was increased at downwind locations (until 150m) and significantly differed from upwind measurements. Although ST398 was mostly located inside farms, in 50-80% of the cases it was also detected at 30m from the barns. In the current research project farms were selected with a maximum of one farm in a radius of 1000m. Also the farm fields sampled are not always located in close vicinity of the farm, reducing the possibility of detecting MRSA in the samples due to farm influence. The German and Dutch studies showed higher downwind than upwind MRSA occurrence. The current study however, cannot conclude on this phenomenon as too little air filter samples were considered positive.

In general, the low amount of estimated gene copies in manure, soil and air filters present a problem for quantification. This experiment demonstrated high amounts of positive samples, but the strict LOQ prevented reliable quantification beyond the range of the C_q cut-off. This fact was also observed when the SKBII samples were initially analyzed with a probe based assay (C_q cut-off ≤ 35), which only confirmed the manure sample findings from farm 4. The latter being probably due to its lower sensitivity already explained in Part I. In addition, few technical replicates per sample were found positive, when using both assay types. Ways to solve the first problem, is to use more template DNA or to run more qPCR cycles, however these methods include more costs, will increase the chance of detecting contamination or even increase the probability of qPCR inhibition. The second finding concerning the qPCR replicates, can be addressed by running multiple replicates e.g. by means of a 'MPN-qPCR'. Nevertheless, the basic MPN condition, the observation of a clear gradual extinction of the qPCR signal throughout the dilution series (with C_qs at least higher than the LOQ), couldn't be met for those samples with a low amount of positive technical replicates. For instance, as presented in Table IV, those particular samples showed some positive replicates at only one DNA concentration and no 100% positivity was noticed when examining the highest DNA concentration. Moreover, also inhibition was observed at the highest DNA concentration. As a result, the MPN assay was not considered useful and no further MPN calculations were or could be made.

5.4. Part III: Conclusion

In conclusion, no additional proof for the accumulation of antibiotic resistance was found in soil and air samples from pig, poultry and calf farms in comparison with the results from the MRSA ST398 qPCR assay (SKBII project). The results merely confirmed the low amount of antibiotic resistance (due to MRSA) in soil and air, except for the manure results from one

farm, which indicated *mecA* presence not related to MRSA ST398. However, no indication for the transmission of antibiotic genes from manure to soil and air could be observed. As all samples were only tested with a qPCR, also death bacterial cells are included in the analysis, which do not contribute to the disease causing environmental bacterial load, except when e.g. MRSA exotoxins (however mostly CA-MRSA associated) are already present in the environment. In fact, it is considered good practice to support qPCR figures with culturing figures, before making a statement about any disease causing potential of the environmental bacteria in question. As no such confirmation was found during the SKBII study in soil samples (N.B. only soil samples were cultured; for details and the discussion on the subject, the reader is referred to the SKBII report), at this moment, no clear conclusions on specific health risks can be drawn from the findings in this report.

Acknowledgements
This report never could have been realized without the help of many. First of all, I would like to express my great appreciation to my supervisor, dr. Heike Schmitt for her enthoustiastic way of coaching and for her insightful comments and additions to my work. In addition, I am really greatful to the IRAS-VPH staff for the nice working atmosphere. More in particular, I want to thank Gerdit Greve, Betty Jongerius-Gortemaker, Gamonsiri Bhumibhamon and Jurriaan Hoekstra for always being available for questions, for their support and collaboration. Also specials thanks go out to Angèle Timan, Ali Eggenkamp and Marco van der Wouden for their assistence in culturing and counting the MRSA and *E. coli* colonies. Furthermore, I want to thank Nena Burger for helping with the NucliSens easyMAG and the QIAquick purification protocol, and for performing a qPCR.

For me, the writing of this report also means the end of a long period of studying. I would like to thank my parents, my sister Anneleen, friends and family and Wouter for their continuous support and love, without which, all this would not have been possible.

Finally, I would like to dedicate my work and studies to my grandfather, Jozef Nuyts, who passed away a couple of years ago. In his remembrance, to never forget to get involved, to be innovative and to dare to be a pioneer. Bedankt vava, voor alles.

Annexes

Annex I: DNA extraction protocols

Protocol 1: Lysostaphine and boiling (Schouls et al., 2009)

1. Take 1 ml from each TSB tube and centrifuge 10 minutes at 7500 rpm.
2. Remove the supernatant and suspend the sediment in 50 µl lysis mix in TE (10 mM Tris HCl, 1 mM EDTA, pH 8) and 100 µg/ml lysostaphine.
3. Incubate for 35 minutes at 37°C and 10 minutes at 95°C.
4. Add 450 µl TE buffer.
5. Centrifuge 5 minutes at 13600 rpm.
6. Transfer the supernatant to a new Eppendorf tube.

Total amount of extracted DNA = 450µl per reaction.

Protocol 2: Boiling protocol (Based on Reischl (Reischl et al., 1994))

1. Take 1 ml from each TSB tube and centrifuge 10 minutes at 7500 rpm.
2. Remove the supernatant and suspend the sediment in 200µl lysis buffer (1% Triton X-100, 0.5% Tween 20, 10 mM Tris HCl, pH 8) and 1 mM EDTA.
3. Incubate for 10 minutes at 100°C.
4. Centrifuge 2 minutes at 13600 rpm and pipette the supernatant to a new Eppendorf tube.

Total amount of extracted DNA = 200µl per reaction.

Protocol 3: DNeasy Blood & Tissue Kit®, Qiagen, Venlo, The Netherlands

1. Take 1 ml from each TSB tube and centrifuge 10 minutes at 7500 rpm.
2. Remove the supernatant and resuspend the sediment in 180µl lysis buffer (= 20 mM Tris-HCl, 2 mM Na_2-EDTA, 1.2% Triton X-100, pH 8 + lysozym 20 mg/ml).
3. Incubate 30 minutes at 37°C.
4. Add 25µl proteinase K (Qiagen) and 200µl AL buffer (Qiagen) and fingerflip the solution.
5. Incubate 30 minutes at 56°C and fingerflip the solution from time to time.
6. Fingerflip the solution 15 seconds.
7. Add 200µl ethanol (96-100%).
8. Pipette the solution into a spin column placed inside a collection tube.
9. Centrifuge 1 minute at 8000 rpm.
10. Remove the flow through and tube.
11. Place the column in a new collection tube.
12. Add 500µl AW1 buffer (Qiagen)
13. Centrifuge 1 minute at 8000 rpm
14. Remove the flow through and tube and place the column on a new collection tube.
15. Add 500µl AW2 buffer (Qiagen).
16. Centrifuge 3 minutes at 13600 rpm.
17. Remove the flow through and tube.
18. Place the column on a new collection tube.
19. Add 50µl Milli-Q water (MQ) and allow it to stand for 1 minute.
20. Centrifuge 1 minute at 8000rpm.
21. Place the column on a new collection tube.
22. Add 200µl MQ and allow it to stand for 1 minute.
23. Centrifuge 1 minute at 8000rpm.
24. Pipette the solution to an Eppendorf tube.

Total amount of extracted DNA = 200µl per reaction.

Used materials

EDTA: VWR International B.V., Prolabo BDH chemicals, Amstersam, The Netherlands
Tris-HCl: Merck, Darmstadt, Germany
Triton X-100: Sigma-Aldrich, Zwijndrecht, The Netherlands
Lysozym: Sigma-Aldrich, Zwijndrecht, The Netherlands
Lysostaphine: Sigma-Aldrich, Zwijndrecht, The Netherlands

Annex II: Purification of qPCR product, using QIAquick PCR Purification Kit, Qiagen (Venlo, The Netherlands)

Starting quantity: 135µl qPCR product.

1. Add 5 volumes of PB buffer to 1 volume of PCR sample, vortex.
2. Pipette the mixture in a spin column placed on top of collection tube.
3. Centrifuge at 13.000 rpm (17.900g) for 30-60 sec.
4. Discard the flow through and place the spin column back on the same tube.
5. Add 0.75 ml PE buffer.
6. Centrifuge at 13.000 rpm (17.900g) for 30-60 sec.
7. Discard the flow through and place the spin column back on the same tube.
8. Centrifuge additional at 13.000 rpm (17.900g) for 60 sec.
9. Place spin column in a clean 1.5 ml Eppendorf tube.
10. Add 30 µl MQ water (pH 7-8.5) to the centre of the membrane
11. Leave for 1 minute.
12. Centrifuge at 13.000 rpm (17.900g) for 60 sec.

Total amount of extracted DNA = 30µl per reaction.

Annex III: Optimization process *mecA* qPCR – Evaluated primer pairs

No.	Reference	ES	CS	qPCR	Forward primer (#base pairs) total length 5'-3'	Reverse primer (#base pairs) total length 5'-3'	Length amplicon	Accession Number (GenBank)	Citations
1	(Francois et al., 2003)	X (mainly air)	X	X	CATTGATCGCAACGTTCAATTT (461-482) 22	TGGTCTTTCTGCATTCCTGGA (539-559) 21	99	X52593	137
2	(Sabet et al., 2007)		X	X	AAAACTAGGTGTTGGTGAAGATATACC (1592-1618) 27	GAAAGGATCTGTACTGGGTTAATCAG (1713-1738) 26	147		7
3	(Pasanen et al., 2010)	X (air, soil)	X	X	GATTATGGCTCAGGTACTGCTATCC (1167-1191) 25	ATGAAGGTGTGCTTACAAGTGCTAA (1236-1212) 25	70		6
4	(Kilic et al., 2010)		X	X	AAATATTATTAGCTGATTCAGGTTAC (1672-1697) 26	CGTTAATATTGCCATTATTTTCTAAT (1776-1751) 26	105		6
5	(Geha et al., 1994)		X	X	GTAGAAATGACTGAACGTCCGATAA (318-342) 25	CCAATTCCACATTGTTTCGGTCTAA (603-627) 25	310		179
6	(De Neeling et al., 1998)	*	X		GTTGTAGTTGTCGGGTTTGG (36-55) 20	CTTCCACATACCATCTTCTTTAAC (348-371) 24	336	X52593	84
7	(Costa et al., 2005)		X	X	GATAAAAAAGAACCTCTGCT (1296-1315) 20	ACTGCCTAATTCGAGTG (1568-1552) 17	273	X52593	44
8	(DelVecchio et al., 1995)		X		TAGAAATGACTGAACGTCCG (179-198) 20	TTGCGATCAATGTTACCGTAG (312-332) 21	154		95
9	(Grisold et al., 2002)		X	X	CTAGGTGTGGTGAAGATATACCA (1596-1619) 23	TGAGGTGCGTTAATATTGCCA (1783-1763) 21	188	X52592	59

No.	Reference	ES	CS	qPCR	**Forward primer** (#base pairs) total length **5'-3'**	**Reverse primer** (#base pairs) total length **5'-3'**	**Length amplicon**	**Accession Number (GenBank)**	**Citations**
10	(Reischl et al., 2000)		X	X	CAAGATATGAAGTGGTAAATGG T (1471-1493) 23	TTTACGACTTGTTGCATACCATC (1879-1857) 23	409	X52593	187
11	(Kearns et al., 1999)		X		CGGTAACATTGATCGCAACGTT CA (455-478) 24	CTTTGGAACGATGCCTAATCTCA T (668-645) 24	214		43
12	(Martinea u et al., 2000)		X		AACAGGTGAATTATTAGCACTT GTAAG (1059-1085) 27	ATTGCTGTTAATATTTTTTGAGT TGAA (1206-1232) 27	174	X52593	143
13	(Mason et al., 2001)	X (sand, sea water)			TCCAGGAATGCAGAAAGACCAA AGC (539-563) 25	GACACGATAGCCATCTTCATGTT GG (1013-1037) 25	499	X52593	68
14	(Murakam i et al., 1991)	X (water samples)	X	X	AAAATCGATGGTAAAGGTTGGC (1282-1303) 22	AGTTCTGCAGTACCGGATTTGC (1793-1814) 22	533		347
15	(Petinaki et al., 2001) (partly based on Murakami)	Only when referred to Muraka mi	X		GGTCCCATTAACTCTGAAG (991-929) 19 ATCGATGGTAAAGGTTGGC (1427-1445) 19	AGTTCTGCAGTACCGGATTTGC (1935-1956) 22	1333 / 530		32
16	(Predari et al., 1991)		X		GGGATCATAGCGTCATTATTC (520-540) 21	AACGATTGTGACACGATAGCC (1026-1046) 21	527		67

No.	Reference	ES	CS	qPCR	Forward primer (#base pairs) total length 5'-3'	Reverse primer (#base pairs) total length 5'-3'	Length amplicon	Accession Number (GenBank)	Citations
17	(Glad et al., 2001) (Modification Predari)		X		Same as Predari	ACGATTGTGACACGATAGCC (without first A)	527		4
18	(Smyth et al., 2001)		X		GCAATCGCTAAAGAACTAAG (693-712) 20	GGGACCAACATAACCTAATA (895-914) 20	222	X52594	35
19	(Salisbury et al., 1997)		X		GGGATCATAGCGTCATTATTC (520-540) 21	AACGATTGTGACACGATAGCC (1026-1046) 21	527		29
20	(Xu et al., 2011)		X		GGCATCGTTCCAAAGAATGT (654-672) 20	CCATCTTCATGTTGGAGCTTT (1007-1027) 21	374		0

Key: Reference = The study which mentions the *mecA* PCR for the first time. ES = In the reference study or studies referring to the reference study, environmental samples were tested with the *mecA* (q)PCR; CS = In the reference study or studies referring to the reference study, clinical samples were tested with the *mecA* (q)PCR. qPCR = The reference study or one of the studies referring to the reference study, mentions the use of *mecA* real-time PCR and a the corresponding probe sequence is specified. Accession Number = This reference study cites an Accession Number from GenBank NCBI associated with of a specific *mecA*/MRSA sequence. Length of the amplicon = the specific PCRs were also evaluated based on the length of the target sequence/amplicon (preferably between 100-150 nucleotides). Citations = The amount of citations in Scopus (http://www.scopus.com/search/form.url) in March 2013. The amount of citations was not considered a leading parameter in evaluating the different studies.

Next to the evaluation based on the data in this table, primers were also examined based on GC content, location of the specific Gs and Cs, the published qPCR protocols, and if mentioned, the related sensitivity and efficiency data. After initially ranking and excluding the first primers, the primer pairs still in the running were evaluated with ClustalX2.1 (check for fitting), Primer-Basic Local Alignment Search Tool (Primer-Blast, NCBI; check for cross-reactivity) and Mfold Web Server (check for secondary structures).

* One study testing i.a. dust samples on MRSA (Gilbert et al., 2012) mentions the use of a *mecA* qPCR. Yet, it is however not clear which primer pair was used (contradicting / erroneous references). Possibly the study refers to this primer pair.

Annex IVa: Spiking the filters: SKC filter series

Bacterium	Spiking concentrations	+1**	0	-1	-2	-3	-4	-5		
MRSA ST398	µl spiked on the filter	1000*	100	100	100	100	100	100		
	Cells per filter	1,0E+07	1,0E+06	1,0E+05	1,0E+04	1,0E+03	1,0E+02	1,0E+01		
	Genes per qPCR	6,0E+05	6,0E+04	6,0E+03	6,0E+02	6,0E+01	6,0E+00	6,0E-01		
E.coli tetW	Dilution			-1	-2	-3	-4	-5	-6	-7
	µl spiked on the filter			10	10	10	10	10	10	10
	Cells per filter			1,0E+05	1,0E+04	1,0E+03	1,0E+02	1,0E+01	1,0E+00	1,0E-01
	Genes per qPCR			2,1E+06	2,1E+05	2,1E+04	2,1E+03	2,1E+02	2,1E+01	2,1E+00
E.coli intI1	Dilution			-1	-2	-3	-4	-5	-6	-7
	µl spiked on the filter			10	10	10	10	10	10	10
	Cells per filter			1,00E+05	1,00E+04	1,00E+03	1,00E+02	1,00E+01	1,00E+00	1,00E-01
	Genes per qPCR			2,10E+05	2,10E+05	2,10E+04	2,10E+03	2,10E+02	2,10E+01	2,10E+00
E.coli CTX-M1	Dilution			-1	-2	-3	-4	-5	-6	-7
	µl spiked on the filter			10	10	10	10	10	10	10
	Cells per filter			1,00E+05	1,00E+04	1,00E+03	1,00E+02	1,00E+01	1,00E+00	1,00E-01
	Genes per qPCR			2,10E+05	2,10E+05	2,10E+04	2,10E+03	2,10E+02	2,10E+01	2,10E+00
E.coli ATCC	Dilution					-2	-2	-2	-2	-2
	µl spiked on the filter					40	40	40	40	40
	Cells per filter					4,0E+04	4,0E+04	4,0E+04	4,0E+04	4,0E+04
#Spiked filter replicates		4	5	6	6	6	6	6	6	5
#Spiked cells		1,0E+07	1,0E+06	4,0E+05	4,0E+04	4,4E+04	4,0E+04	4,0E+04	4,0E+04	4,0E+04

#Spiked filters
50

Key:
Spiking concentrations: 1 (= 10x original DNA concentration), 0 (= original DNA concentration), -1 (= 10^{-1} diluted concentration) until -7 (= 10^{-7} diluted concentration).
* The highest spiking concentration was achieved by using a higher volume addition of undiluted TSB.
**For MRSA, all prepared spiking concentrations were derived from the same inoculated TSBs (spiking series A: MRSA inoculated TSB containing oxacillin), except for the highest SKC concentration, which was prepared from 'spiking series B'. Spiking series B itself was prepared from MRSA inoculated TSBs containing oxacillin and MRSA inoculated 'regular' TSBs.

Annex IVb: Spiking the filters: Pall filter series

Bacterium	Spiking concentrations	+1	0	-1	-2	-3	-4	-5		
MRSA ST398	µl spiked on the filter	1000*	100	100	100	100	100	100		
	Cells per filter	1,0E+07	1,0E+06	1,0E+05	1,0E+04	1,0E+03	1,0E+02	1,0E+01		
	Genes per qPCR	6,0E+05	6,0E+04	6,0E+03	6,0E+02	6,0E+01	6,0E+00	6,0E-01		
E.coli tetW	Dilution			-1	-2	-3	-4	-5	-6	-7
	µl spiked on the filter			10	10	10	10	10	10	10
	Cells per filter			1,0E+05	1,0E+04	1,0E+03	1,0E+02	1,0E+01	1,0E+00	1,0E-01
	Genes per qPCR			2,1E+06	2,1E+05	2,1E+04	2,1E+03	2,1E+02	2,1E+01	2,1E+00
E.coli intI1	Dilution			-1	-2	-3	-4	-5	-6	-7
	µl spiked on the filter			10	10	10	10	10	10	10
	Cells per filter			1,00E+05	1,00E+04	1,00E+03	1,00E+02	1,00E+01	1,00E+00	1,00E-01
	Genes per qPCR			2,10E+05	2,10E+05	2,10E+04	2,10E+03	2,10E+02	2,10E+01	2,10E+00
E.coli CTX-M1	Dilution			-1	-2	-3	-4	-5	-6	-7
	µl spiked on the filter			10	10	10	10	10	10	10
	Cells per filter			1,00E+05	1,00E+04	1,00E+03	1,00E+02	1,00E+01	1,00E+00	1,00E-01
	Genes per qPCR			2,10E+05	2,10E+05	2,10E+04	2,10E+03	2,10E+02	2,10E+01	2,10E+00
E.coli ATCC	Dilution					-2	-2	-2	-2	-2
	µl spiked on the filter					40	40	40	40	40
	Cells per filter					4,0E+04	4,0E+04	4,0E+04	4,0E+04	4,0E+04
#Spiked filter replicates		4	4	4	6	6	6	4	4	4
#Spiked cells		4,0E+00	4,0E+00	4,0E+00	4,0E+00	3,0E+00	3,0E+00	4,0E+00	4,0E+00	4,0E+00

#Spiked filters
42

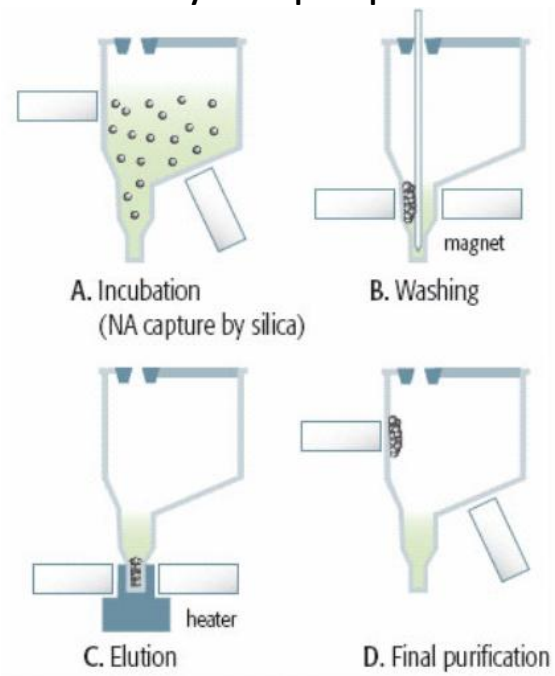
Key:
Spiking concentrations: 1 (= 10x original DNA concentration), 0 (= original DNA concentration), -1 (= 10^{-1} diluted concentration) until -7 (= 10^{-7} diluted concentration).
* The highest spiking concentration was achieved by using a higher volume addition of undiluted TSB.
All prepared spiking concentrations were derived from the same inoculated TSBs (spiking series A: MRSA inoculated TSB containing oxacillin).

Annex V: NucliSENS® easyMAG® protocol (Biomérieux Clinical Diagnostics, Marcy l'Etoile, France)

1. Place the tube in the magnetic holder and wait 10 seconds for the beads to move to the magnets.
2. Discard the fluid in a waste container and make sure that there is still ~1 ml fluid left.
3. Resuspend the beads in the remaining ~1 ml fluid and transfer it to a 1,5 ml Eppendorf tube in the magnetic holder.
4. Wait again for the beads to move to the magnet and discard the remaining fluid with a pipette.
5. Release the samples from the magnet and add 400 µl of Extraction Buffer 1 (EB1) to your samples.
6. Place the samples back in the magnet holder and wait for the beads to move to the sides and discard all fluid.
7. Remove the samples from the magnet holder and add 400µl Extraction Buffer 2 (EB2) and resuspend.
8. Place the samples back in the magnet holder and wait for the beads to move to the sides and discard all fluid.
9. Remove the samples from the magnet holder and add again 400 µl Extraction Buffer 2 (EB2) and resuspend.
10. Place the samples back in the magnet holder.
11. Wait again for the beads to move to the magnet holder and discard the remaining fluid with a pipette.
12. Remove the Eppendorf tube from the magnetic holder and add 400 µl Extraction Buffer 3 (EB3).
13. Place the Eppendorf tube back in the magnetic holder and wait for all the beads to move to the side.
14. Remove the remaining fluid with a pipette. Caution: Steps 12-14 may only take 15 seconds to prevent the eluting reaction to start.
15. Remove the Eppendorf tube from the magnetic holder and add 50 µl EB3 and collect the beads to the bottom.
16. Incubate the samples in a thermomixer at 1100 rpm for 5 minutes at 60°C (Eppendorf Thermomixer® comfort, Hamburg, Germany).
17. Place the samples back in the magnetic holder and wait for all the beads to move to the side.
18. Pipette the remaining fluid containing the DNA to another Eppendorf tube.

Total amount of extracted DNA = 50µl per reaction

NucliSENS® easyMAG® principle.



Source: http://www.biomerieux-diagnostics.com

Annex VI: Positive manure samples by farm and subsample

Farm (1-16) manure subsample (a-b)	#Positive replicates / total qPCR replicates (10x diluted DNA)	#*mecA* gene copies/kg manure (C_qs ≤ 32,5)	Mean #*mecA* gene copies/kg manure (C_qs ≤ 32,5)	Animal origin of the manure used
1a	2/8*	1,27E+07	1,42E+07	Pigs
1b	3/8*	1,58E+07		
2a				Pigs
2b				
3a	1/8			Poultry
3b	8/8	1,30E+08		
4a	8/8	9,68E+09	1,29E+10	Poultry + Pigs
4b	8/8	1,62E+10		
5a	1/8			Pigs
5b	-*	1,47E+07		
6a	1/8			Pigs
6b				
7a	1/8**			Pigs
7b	3/8**	1,41E+08		
8a	1/8	1,31E+08		Pigs
8b				
9a				Pigs
9b				
10a				Calves
10b				
12a				Pigs
12b				
13a	2/8*			Pigs
13b	4/8*			
14a	1/8	1,48E+07		Pigs
14b	1/8			
15a	1/8			Pigs
15b	2/8			
16a	2/8			Pigs
16b	1/8*	1,80E+07		

* = All samples were positive in the original (1x) DNA concentration.

**= Two out of three samples were positive in the original (1x) DNA stock concentration.

N.B.: Manure samples from farm 11 (pig manure) were not included in the study due to lack of original DNA.

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List of abbreviations

A. fumigates: *Aspergillus fumigatus*
ATCC: American Type Culture Collection
B. atrophaeus: *Bacillus atrophaeus*
CA-MRSA: Community-Associated MRSA
CC398: Clonal Complex 398
CFU(s): Colony Forming Unit(s)
C_q: qPCR quantification cycle
CV(s): Coefficient(s) of Variation
DNA: Deoxyribonucleic acid
dNTP (dATP, dCTP, dGTP, dTTP): deoxyribonucleotide triphosphates (adenosine, cytidine, guanosine, thymidine)
dsDNA: double stranded DNA
E. coli: *Escherichia coli*
ESBL(s): Extended Spectrum Beta-Lactamase(s)
GMO: Genetically Modified Organism
GLM: General linear model
HA-MRSA: Hospital-Acquired/Associated MRSA
IRAS: Institute for Risk Assessment Sciences, Utrecht University, The Netherlands
LOD: Limit of Detection
LOQ: Limit of Quantification
MDL: Method Detection Limit
mecA: antibiotic resistance gene in i.a. *Staphylococcus aureus* coding for 'methicillin resistance'
MLST: Multi-locus Sequence Typing
MLVA: Multi-locus Variable-Number Tandem Repeat Analysis
MQ: Milli-Q water
MRCNS: Methicillin Resistant Coagulase Negative Staphylococci
MRSA: Methicillin Resistant *Staphylococcus aureus*
MRSA ST398: MRSA Sequence Type 398
MRSA *tetM*+: MRSA AP009324 *tetM*+
MRSA unknown: Unknown MRSA strain, probably MRSA ST398
NCBI: National Center for Biotechnology Information
NTC: Non-Template control
(q)PCR: (Real-time) Polymerase Chain Reaction
PDU(s): PCR Detectable Unit(s)
PGFE: Pulsed-Field Gel Electrophoresis, Macro-restriction pattern analysis
PM: Particulate matter
Primer-BLAST: Primer-Basic Local Alignment Search Tool, NCBI
PTFE: Polytetrafluoroethylene (e.g. Teflon)
PVL: Panton-Valentine leukocidin exotoxin
RFU(s): qPCR Relative Fluorescence Unit(s)
SCC*mec* (typing): Staphylococcal Cassette Chromosome *mec* (typing)
SKBII study: 'Antibiotic resistance in soil part II study – Stichting Kennisontwikkeling Kennisoverdracht Bodem II'
spa typing: *Staphylococcus aureus* protein A gene (*spa*) typing
T_a: Annealing temperature
T_m: Melting temperature
TSA: Trypticase Soy Agar
TSB: Trypticase Soy Broth

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