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TNO report

TNO-DV2 2005 A165

Fast detection of ciprofloxacin resistance, part I

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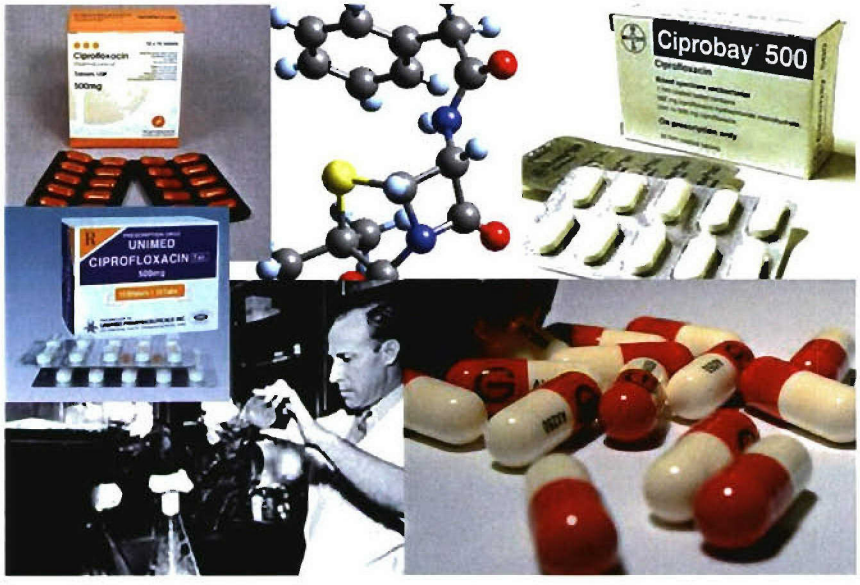
Snelle detectie van ciprofloxacine resistentie, deel I

In opdracht van het Ministerie van Defensie werd onderzoek gedaan naar methoden voor het aantonen van eventueel aanwezige resistentie eigenschappen in bacteriën. Het onderzoek is uitgevoerd in de business unit 'Biologische en Chemische Bescherming' van TNO Defensie en Veiligheid. Het onderzoek is gefinancierd in het kader van programma V013 en hoort bij resultaatnummer 807b.



Organisatie en financiering

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Probleemstelling

Wanneer antibioticum resistentie (bijvoorbeeld tegen ciprofloxacine) vroegtijdig herkend wordt kan een meer effectieve therapie worden toegepast, en kunnen mogelijk levens worden gered. Het hier beschreven onderzoek bouwt voort op hetgeen beschreven is in rapport DV2 2005-A050, en beschrijft uitbreiding en verbetering van de analysemethode.

Beschrijving van de werkzaamheden

Twee gepubliceerde methoden voor snelle detectie van ciprofloxacine-resistentie werden geëvalueerd met behulp van de eerder verkregen (rapport DV2 2005-A050) resistente stam van *Serratia marcescens*. Om de methode ook op een Gram-positieve bacterie te kunnen toepassen werd een niet-virulente stam van *Bacillus anthracis* resistent gemaakt. Vervolgens werd de snelle detectiemethode ook hierop toegepast. Als extra aspect werd een serie stammen van de beruchte MRSA-bacterie (methicilline resistente *Staphylococcus aureus*, die tevens resistent waren tegen een reeks andere antibiotica) geanalyseerd met een commerciele kit voor detectie van MRSA en werden DNA-mutaties in kaart gebracht die geassocieerd zijn met ciprofloxacin-resistentie.

Resultaten en conclusies

Van de twee gepubliceerde methoden voor het detecteren van ciprofloxacine-resistentie bleek de methode volgens Lindler de beste. In de publicatie werd deze methode gebruikt voor Yersinia pestis. In dit onderzoek werd de methode met succes geschikt gemaakt voor Serratia marcescens en Bacillus anthracis. Hiermee werd aangetoond dat de methode breed toepasbaar is en betrouwbaar werkt.
De methode heeft een analysetijd van minder dan twee uur. De commerciele MRSA-kit voldeed goed. Omdat de MRSA-kit met dezelfde technologie (realtime PCR)

werkt als de ciprofloxacine-resistentiebepaling kan dit eenvoudig gecombineerd worden. De DNA-mutaties die geassocieerd zijn met ciprofloxacine-resistentie blijken een consistent patroon te volgen, hetgeen van belang is voor verdere uitbreiding van de methode naar meerdere bacteriesoorten.

Toepasbaarheid

De methode voor het detecteren van ciprofloxacine-resistentie is toepasbaar in situaties waarin de bacteriesoort reeds geidentificeerd is. Direct daarna kan worden vastgesteld of resistentie aanwezig is. Indien dit het geval blijkt kan kostbare tijd gewonnen worden, en kunnen mogelijk zelfs levens gered worden, door een ander antibioticum toe te passen. De methode is breed toepasbaar, waarschijnlijk voor alle bacteriesoorten. De methode is in de huidige vorm in bijvoorbeeld een veldlaboratorium toepasbaar, mits daarvoor geschikte apparatuur (met name realtime PCR) aanwezig is.

Vervolgafspraken

De ontworpen methode zal verder worden verbeterd, en geschikt worden gemaakt voor een groter aantal bacteriesoorten, eerst nog in het kader van programma V013, daarna V502.

PROGRAMMA	PROJECT
Programmabegeleider A.S. de Koning, arts, MGFB	Projectbegeleider Dr. H.J. Jansen, MGFB
Programmaleider Dr. M.S. Nieuwenhuizen, TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming	Projectleider ir. M.P. Broekhuijsen, TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming
Programmatitel Passieve verdediging tegen NBC-wapens	Projecttitel Antibiotica resistentie screening
Programmanummer V013	Projectnummer 014.14001
Programmaplanning Start 2001 Gereed 2005	Projectplanning Start 2001 Gereed 2005
Frequentie van overleg Met de programma/projectbegeleider werd twee maal per jaar gesproken over de invulling en de voortgang van het onderzoek.	Projectteam M.P. Broekhuijsen en W.C.M. van Dijk, TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming

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Summary

Organisation and financing
By order of the Dutch Ministry of Defence, a study was performed to develop or improve methods for the detection of antibiotic resistance in bacteria. The study was performed in the Business Unit 'Biological and Chemical Protection' of TNO Defence, Security and Safety. The study was financed under the program 'Protection against NBC weapons'.

Background and goal
When antibiotic resistance (e.g. against ciprofloxacin) is detected timely, a more successful therapy can be adopted, and possibly lives can be saved. The current study follows on a previous one that is described in TNO report DV2 2005-A050, and describes improvements of the method.

Experimental study
Two published methods for fast detection of ciprofloxacin resistance were evaluated using a resistant strain of *Serratia marcescens*. To test the method on a Gram-positive strain, a non-virulent strain of *Bacillus anthracis* was made resistant against ciprofloxacin, and potential mutations in three genes were analysed. Resistant mutants were used to validate the method. Several strains of MRSA bacteria (methicillin resistant *Staphylococcus aureus*, which were also resistant to several other antibiotics) were analysed using a commercial kit for fast detection of MRSA and screened for mutations associated with ciprofloxacin-resistance.

Results and conclusions
Of the two published methods for detecting ciprofloxacin resistance, the one by Lindler appeared to be the best. This method was published using *Yersinia pestis* as target. During the study, the method was adapted to *Serratia marcescens* and *Bacillus anthracis*, whereby it was shown that the method is reliable and suitable for many (perhaps all) bacterial species. The method is performed within two hours. The commercial MRSA kit performed well. Because this kit uses the same technology (realtime PCR) as is used for detecting ciprofloxacin resistance, it can easily be used in combination. DNA mutations associated with ciprofloxacin resistance appear to follow a consistent pattern, which is important for further extension of the method to other bacterial species.

Applicability
The method for detecting ciprofloxacin resistance can be applied directly after the bacterial species has been identified, and within two hours after that. If resistance is detected, valuable time can be saved, or even lives, by applying another antibiotic. The method can be applied to a broad range of bacteria, probably to all bacterial species. The method is applicable in field laboratories, assuming the presence of real-time PCR equipment.

Further study
The method will be further improved, and extended to other relevant bacterial species, under the same program of the Ministry of Defence.

Contents

Abbreviations

ATCC	American Type Culture Collection
bp	base pair(s)
DNA	Deoxyribo-Nucleic Acid
dNTP	deoxy-Nucleoside-Tri-Phosphate
EtBr	Ethidium bromide
FRET	Fluorescence Resonance Energy Transfer
grlA	topoisomerase IV, subunit A (or parC)
gyrA	topoisomerase II, subunit A (or gyrase A gene)
LB	Luria broth
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
PCR	Polymerase Chain Reaction
RIVM	National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu)
RNA	Ribo-Nucleic Acid
TSA	Trypticase soy agar
QRDR	Quinolone resistance determining region

1 Introduction

1.1 Goal and approach of the project

The goal of the project is to investigate, develop, improve, or implement methods for the detection of antibiotic resistance in bacteria.

Most currently used methods for screening antibiotic resistance in a strain rely on culturing in the presence of antibiotics (Figure 1). Several commercial systems exist for this purpose. These methods were not explored or evaluated. The aim was to develop DNA-based methods, which are inherently faster than conventional methods and potentially more suitable for field use.

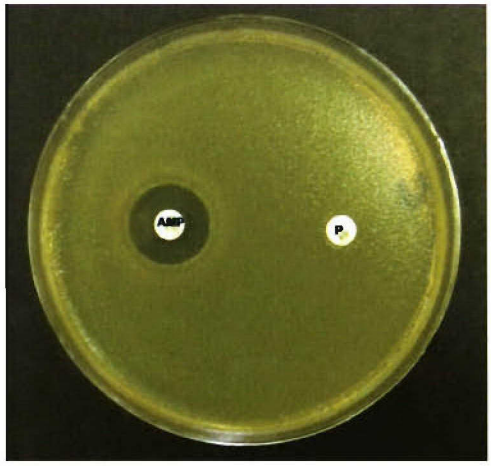

Figure 1 Example of antibiotic resistance testing using culture media. *Escherichia coli* bacteria are cultured as a layer on an agar plate. The susceptibility of the bacteria towards certain antibiotics is shown by applying a drop of antibiotic on the agar plate before culturing, resulting in a circular clear region (AMP) where bacteria will not grow. Resistant bacteria will multiply regardless of the presence of antibiotic (P). AMP: Ampicillin, P: penicillin.

The study is primarily directed towards operationally relevant antibiotics and strains, i.e. antibiotics that are commonly used by military personnel, and bacterial species that are considered to be biowarfare agents, or that are likely to be encountered by military forces during operations.

1.2 Background

When observing bacterial infections in patients, or potentially pathogenic bacteria in environmental samples, the immediate need is to identify the bacteria. Once knowing the identity of the bacteria, one can prepare appropriate medical countermeasures. The immediately following concern is whether the identified bacteria carry any antibiotic resistance. Most bacterial infections can be treated effectively using antibiotic compounds, provided they are in sufficient stock and administered timely (early in the infection process), and provided the bacterial organisms are susceptible to the antibiotic used. However, different bacterial species can vary in their susceptibility towards different antibiotic compounds. Even worse, many bacterial strains have developed resistance against specific antibiotics. Nowadays, many antibiotic compounds are

known, both naturally occurring and of synthetic origin, which fall into several classes, based on mechanism and action. Resistance against all known antibiotics has been described.

In a previous report, general background information concerning the discovery of antibiotics, the development of resistance, and the mechanism of action and resistance have been described (Broekhuijsen and van Dijk 2005). In this previous study, experiments were described to detect antibiotic resistance associated with two types of resistance genes. One of the two methods, using conventional PCR, was aimed at detecting acquired resistance genes, e.g. genes that confer resistance against penicillin or ampicillin. The other method was aimed at detecting ciprofloxacin resistance, and used a combination of conventional PCR and DNA sequencing to detect mutations that are associated with ciprofloxacin resistance. The latter method worked well, but has an analysis time of over 6 hours. In addition, conventional PCR is less suitable for use in the field, mainly because of the required gel electrophoresis step.

In this report, experiments are described to improve the method for detecting ciprofloxacin resistance, using *real-time* PCR. The improved method uses the so-called mutation assay of the LightCycler equipment, where hybridisation probes are used in combination with ordinary PCR primers (Broekhuijsen and Boomaars, 2001). The method is based on one of two published methods that seemed promising for this purpose. The aim of the current study was to simplify the method described in the previous report, as well as decreasing the time required to perform the method. In addition to improving the method, it was also extended to other bacterial species.

In addition, a commercially available kit for fast detection of MRSA bacteria was tested, because MRSA bacteria are relevant pathogens in a clinical setting. The MRSA strains are also ciprofloxacin resistant, and thereby provided an excellent opportunity to test the validity and applicability of the improved method for detecting ciprofloxacin resistance.

2 Materials and methods

2.1 Bioinformatics

Genes or DNA sequences were searched in Genbank on the NCBI website. Specific genes of interest were downloaded and analysed using the software package DNAMAN version 5.2.9 (Lynnon Biosoft, Canada). Primers were designed using the primer-design tool in DNAMAN, with 60+/-2 °C as standard annealing temperature. Probes were designed by comparing the probes used in public literature with the target sequence, and adapted accordingly. DNA and protein sequences were aligned using the Multiple Sequence Alignment tool in DNAMAN with the default parameters.

2.2 Bacterial strains

The strains listed in Table 1 were used in this study.

Table 1 Strains used in this study. 'TNO-code' refers to the strain collection at TNO Defence, Security and Safety, location Rijswijk. 'Other code' refers to codes used by supplying organisation.

Species	TNO-code	Other codes	Comment
Serratia marcescens	BM107	ATCC13880	type-strain, cipro-sensitive
Serratia marcescens	BM108	ATCC43297-M	mutant, cipro-resistant
Bacillus anthracis	BM232	ATCC4229	non-virulent, pXO1-/pXO2+
Staphylococcus aureus	BM187	ATCC29213	cipro-sensitive
Staphylococcus aureus	BM292	RIVM02-00855	MRSA, cipro-sensitive
Staphylococcus aureus	BM293	RIVM02-00664	MRSA, cipro-resistant
Staphylococcus aureus	BM294	RIVM02-00279	MRSA, cipro-resistant
Staphylococcus aureus	BM295	RIVM02-00578	MRSA, cipro-resistant
Staphylococcus aureus	BM296	RIVM02-00880	MRSA, cipro-resistant
Staphylococcus aureus	BM297	RIVM01-01177	MRSA, cipro-resistant
Staphylococcus aureus	BM298	RIVM02-00679	MRSA, cipro-resistant

Both strains of *S. marcescens* were purchased from ATCC. *B. anthracis* ATCC4229 was kindly provided by dr. Mats Forsman from the Swedish Defence Research Agency (FOI) in Umeå, Sweden. *S. aureus* strain BM187 was kindly provided by a local hospital. All other *S. aureus* strains were kindly provided by dr. Wieger Homan from the Dutch National Institute for Public Health and the Environment (RIVM) in Bilthoven, The Netherlands. All strains were cultured in LB liquid medium or on TSA plates, at 37 °C.

Thermolysates were prepared by heating 1 ml of overnight culture in LB at 95 °C for 20 minutes. Thermolysates were analysed for any residual presence of live agent by plating out on TSA and culturing for 5 days at 37 °C. Inactivated thermolysates were used for conventional as well as real-time PCR, mostly after 1:1000 dilution in water.

2.3 Conventional PCR and gel electrophoresis

Conventional PCR was performed using an MJ Research PTC-200 thermocycler, standard PCR tubes of 0.5 ml volume, with a reaction mix volume of 50 µl. Taq-polymerase and dNTP-mix was used from Roche Diagnostics. End concentrations in

the PCR reaction mixture were 2.5 units Taq-polymerase, 1.5 mM MgCl2, 0.2 µM primers, 1 mM dNTP. A standard program was used with the following parameters:
1 5 min. 92 °C;
2 30 sec. 92 °C;
3 30 sec. 60 °C;
4 30 sec. 72 °C
5 goto 2, 34 times;
6 5 min. 72 °C;
7 15 °C for ever;
8 end.

After PCR, a 10 µl sample was analysed on a standard 1.2% agarose gel in 0.5x TBE buffer (0.89 M Tris borate, 0.02 M EDTA, pH 8.3). Loading buffer was used according to Sambrook et al (1989). Electrophoresis was performed during 45 minutes at 100 mA. Gels were visualized using EtBr. Molecular weight markers used was M-9 (digest of DNA from ΦX174 with HinfI, obtained from Eurogentec), containing DNA fragments of 726, 713, 553, 500, 427, 417, 413, 311, 249, 200, 151, 140, 118, 100, 82, 66, 48, 42, 40, 24 bp.

2.4 Realtime PCR

Real-time PCR was performed using the LightCycler (Roche Diagnostics), with a reaction volume of 20 µl. Reagents were used from Roche Diagnostics, e.g. the DNA Master SYBR Green I kit and the DNA Master HybProbe kit. Hybridisation probes were obtained from TibMolbiol in Berlin, Germany. Walker's method was performed essentially according to Walker et al. (2001), and Lindler's method according to Lindler et al. (2001), except that primer and probe sequences were adapted to the *gyrA* gene of *S. marcescens*, and very minor modifications of the program parameters.

Although many details differ between the two methods, not all are relevant. The essential difference between Walker's and Lindler's methods is the use of one probe together with SybrGreen (by Walker) or two probes (by Lindler). In addition, the Mg^{2+} concentration differs, which might influence efficiency. This was not optimised in this study, however. For further details on the methods of Walker and Lindler, see paragraphs 3.1.1 and 3.1.2.

PCR reaction mix for Walker's method:
2.0 µl forward primer GyrA-F01 (10 µM);
2.0 µl reverse primer GyrA-R01 (4 µM);
2.0 µl probe GyrA-P03 (5 µM);
2.0 µl DNA sample from *Serratia marcescens;*
1.6 µl $MgCl_2$ (25 mM);
2.0 µl mastermix SybrGreen I (with 10 mM $MgCl_2$);
8.4 µl H_2O;
End concentration $MgCl_2$ = 3 mM.

The PCR reaction conditions according to Walker's method are described in Table 2.

Table 2 Program parameters used for Walker's method.

Segment number	Temperature Target (°C)	Hold time (sec)	Slope (°C/sec)	Acquisition mode
Program:	Denaturation			Cycles:1
1	95	300	20	none
Program:	Amplification			Cycles:40
1	95	0	20	None
2	60	5	20	None
3	72	30	20	Single
Program:	Amplification2			Cycles:2
1	72	60	20	Single
Program:	Melting			Cycles:1
1	99	30	20	None
2	45	0	20	None
3	95	0	0.2	Continuous
Program:	Cooling			Cycles:1
1	40	30	20	None

PCR reaction mix for Lindler's method:
2.0 µl forward/reverse primers GyrA-F07/-R07 (each 10 µM);
2.0 µl probes GyrA-P01/-P02 (each 4 µM);
2.0 µl DNA sample from *Serratia marcescens;*
3.2 µl $MgCl_2$ (25 mM);
2.0 µl Hot start reaction mix (with 10 mM $MgCl_2$);
8.8 µl H_2O;
End concentration $MgCl_2$ = 5 mM.

The PCR reaction conditions according to Lindler's method are described in Table 3.

Table 3 Program parameters used for Lindler's method.

Segment number	Temperature Target (°C)	Hold time (sec)	Slope (°C/sec)	Acquisition mode
Program:	Denaturation			Cycles:1
1	95	600	20	None
Program:	Amplification			Cycles:40
1	95	10	20	None
2	58	15	20	Single
3	72	15	20	None
Program:	Amplification2			Cycles:2
1	72	30	20	None
Program:	Melting			Cycles:1
1	95	0	20	None
2	40	30	20	None
3	95	0	0.1	Continuous
Program:	Cooling			Cycles:1
1	40	30	20	None

The LightCycler MRSA Detection Kit for the detection of the Methicillin resistance gene (mecA) was purchased from Roche Diagnostics (Cat. No. 3 335 038) and used according to the manufacturers instructions. Thermolysates were diluted 1:1000 in water, and 5 µl of the dilution was used in the reaction mix.

2.5 Primers and probes

Properties of primers and probes are summarised in Table 4. Primers for the *gyrA* gene were designed for *S. marcescens*, *S. aureus*, and *B. anthracis*. Primers for the *grlA* gene were designed for *S. aureus* and *B. anthracis*. Probes were designed for the *gyrA* gene of *S. marcescens*, and for the *gyrA* and *grlA* gene of *B. anthracis*. Primers for *S. aureus* (for both *gyrA* and *grlA*) were used only for obtaining amplicons for sequence analysis. No probes were designed for *S. aureus*.

Table 4 Primer and probe data. The name of the primer or probe reflects the target gene (gyrA or grlA). Primers are either forward (F) or reverse (R). Probes are designated with P. n.a.: not applicable. Target organisms: Sma = *S. marcescens*, Sau = *S. aureus*, Ban = *B. anthracis*.

Name	Sequence (5'-3')*	Label (5')	Label (3')	Target organism
GyrA-F01	TC(CG)TATCTGGA(CT)TATGCGATGTC	n.a.	n.a.	Sma
GyrA-R01	CGCACTTCGGTATAACGCAT	n.a.	n.a.	Sma
GyrA-F07	GACTATGCGATGTCCGTTATTGTC	n.a.	n.a.	Sma
GyrA-R07	GAAGTTACCCTGACCGTCCAC	n.a.	n.a.	Sma
GyrA-F16	AATGAACAAGGTATGACACC	n.a.	n.a.	Sau
GyrA-R16	TACGCGCTTCAGTATAACGC	n.a.	n.a.	Sau
GyrA-F19	ATGTCAGACAATCAACAACAAGC	n.a.	n.a.	Ban
GyrA-R19	ACATTCTTGCTTCTGTATAACGC	n.a.	n.a.	Ban
GrlA-F01	ACTTGAAGATGTTTTAGGTGAT	n.a.	n.a.	Sau
GrlA-R01	TTAGGAAATCTTGATGGCAA	n.a.	n.a.	Sau
GrlA-F09	CGTGACGGCTTAAAACCAGTA	n.a.	n.a.	Ban
GrlA-R09	CTTCCGTATAACGCATTGCTG	n.a.	n.a.	Ban
GyrA-P01	GCACGGTGACAGCGCGGTT	n.a.	Fluorescein	Sma
GyrA-P02	ACGACACTATCGTGCGTATGGCTCA	Red640	n.a.	Sma
GyrA-P03	AGTGTCGTAAACCGCGCTGT	Cy5	Bio	Sma
GyrA-P15	GATTCAGCTGTTTATGAAACGATGGTACG	n.a.	Fluorescein	Ban
GyrA-P16	ATGGCGCAAGATTTCAGTCAACGTT	Red640	n.a.	Ban
GrlA-P03	CCGCACGGTGATTCCTCTGT	n.a.	Fluorescein	Ban
GrlA-P04	TATGAAGCGATGGTACGTTTAAGTCA	Red640	n.a.	Ban

* Nucleotides within brackets represent degenerate positions, with a mix of both nucleotides at that position, e.g. (CG) denotes C or G at a single position.

2.6 DNA manipulations and sequencing

DNA isolation for PCR was necessary for *S. marcescens* and was done using the DNA isolation kit from PureGene, according to the manufacturers instructions. For other strains, crude thermolysates were suitable for PCR. For DNA sequencing of amplicons, the GeneClean Spin kit was used instead, according to the manufacturers instructions. DNA sequencing was performed by the company Baseclear in Leiden, using their standard procedures. PCR amplicons and PCR primers were sent to Baseclear for sequencing. Resulting DNA sequences were received from Baseclear by email.

3 Results

The experimental study was aimed at developing a fast DNA-based method for the detection of ciprofloxacin resistance. Two published methods for this (Walker et al., 2001 and Lindler et al., 2001) were evaluated, and the best performing method was adapted to other bacterial species. A non-virulent strain of *Bacillus anthracis* (obtained from FOI, Sweden) was made resistant against ciprofloxacin, and used for validation of the method, together with a resistant strain of *Serratia marcescens* that was obtained earlier (Broekhuijsen and van Dijk, 2005). Several MRSA strains were analysed with a commercial kit (based on real-time PCR) for detection of MRSA bacteria. The MRSA strains were also analysed for possible mutations related to ciprofloxacin resistance.

The experimental work is schematically summarized below:

1 Evaluation of two published methods for detecting ciprofloxacin resistance (paragraph 3.1).
2 Induction of ciprofloxacin resistance in a non-virulent strain of *Bacillus anthracis* and DNA sequence analysis of obtained ciprofloxacin resistant (mutant) strains (paragraph 3.2).
3 Use of Lindler's method on *Bacillus anthracis* (paragraph 3.3).
4 Analysis of MRSA strains (paragraph 3.4).

3.1 Published methods for detecting ciprofloxacin resistance

After screening the public literature for suitable methods for detecting resistance against ciprofloxacin, two publications seemed relevant. Walker et al. (2001) described a method using real-time PCR with a so-called hybridisation probe which they used on *Salmonella enterica*. Lindler et al. (2001) described a similar method, however using two neighbouring hybridisation probes, which they used on *Yersinia pestis*. Both authors used the LightCycler as platform and a fluorescence detection principle that is called Fluorescence Resonance Energy Transfer (FRET), in combination with a melting point analysis. Using real-time PCR with the LightCycler has been described earlier (Broekhuijsen and Boomaars, 2001).

Using FRET in combination with a melting point analysis on the LightCycler is very suitable for fast and sensitive detection of point mutations in DNA. This is precisely the reason why it is attractive for detecting ciprofloxacin resistance, because resistance is mostly based on (known) point mutations in a limited region of DNA (Broekhuijsen and van Dijk, 2005).

For clarity, the basic method and both variations (i.e. according to Walker et al. and to Lindler et al.) will be shortly explained below. Explaining the method is done in three steps (paragraphs 3.1.1 through 3.1.3). These can be skipped if the reader is familiar with real-time PCR and melting point analysis. Paragraph 3.1.4 describes the experimental evaluation of both published methods, using *Serratia marcescens* as a model organism.

3.1.1 What is FRET?

Fluorescence Resonance Energy Transfer (FRET) is the transfer of energy from one fluorophore (FP) to another (Figure 2). One fluorophore (FP-1) is activated using a suitable wavelength (WL): WL-1. The energy absorbed by the fluorophore can be

measured directly by reading at another wavelength (WL-2) which is emitted by FP-1, but this is not useful in this case. However, when the other fluorophore (FP-2) is in close enough proximity to FP-1, the energy is then transferred from FP-1 to FP-2. This process is called FRET, and can be measured by reading out at still another wavelength (WL-3) which is only emitted by FP-2 upon activation by FP-1.

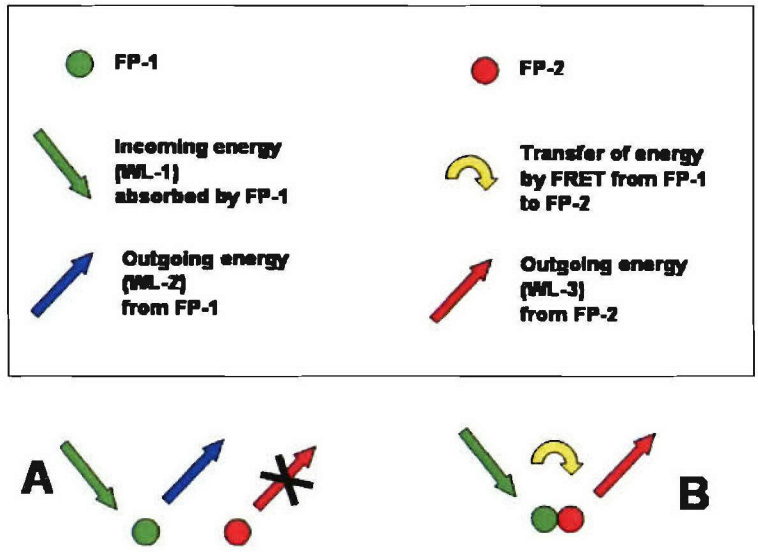

Figure 2 Principle of FRET. Left (A): The two fluorophores FP-1 and FP-2 are not in close proximity, and FRET does not occur. Right (B): FP-1 and FP-2 are in close proximity, thereby permitting FRET, which is measured by reading at wavelength 3 (WL-3).

3.1.2 *Detection of the amplified DNA target region*

In real-time PCR, the DNA target region is being amplified using ordinary primers, just as in conventional PCR. One of the additional features in real-time PCR is the use of one or two probes. The probe(s) can attach to part of the target sequence, according to their design. However, at the start of the PCR reaction, only very little target DNA is present, and almost all probe molecules are floating free in the reaction mixture. After sufficient amplification, more probe molecules attach to the target sequence. Only then FRET can occur, because FRET is dependent on very close proximity of the two fluorophores used (Figure 2). FRET is measured by reading the wavelength that is emitted by the second fluorophore. This outcoming signal will increase during the amplification, and is a measure of the original presence of the specific target DNA sequence.

Two variations of this principle are used by Walker et al. (2001) and Lindler et al. (2001), respectively. Figure 3 shows the variation used by Walker et al. In this case, SybrGreen I (SG1) functions as FP-1. SG1 is often used for aspecific measurement of amplification, because it binds preferentially to double-stranded DNA, and only then it emits fluorescence (Broekhuijsen and Boomaars, 2001). In this variation, the probe carries Cy5, which functions as FP-2. When more target DNA is being amplified, more probe molecules bind to the target region, and more signal is measured.

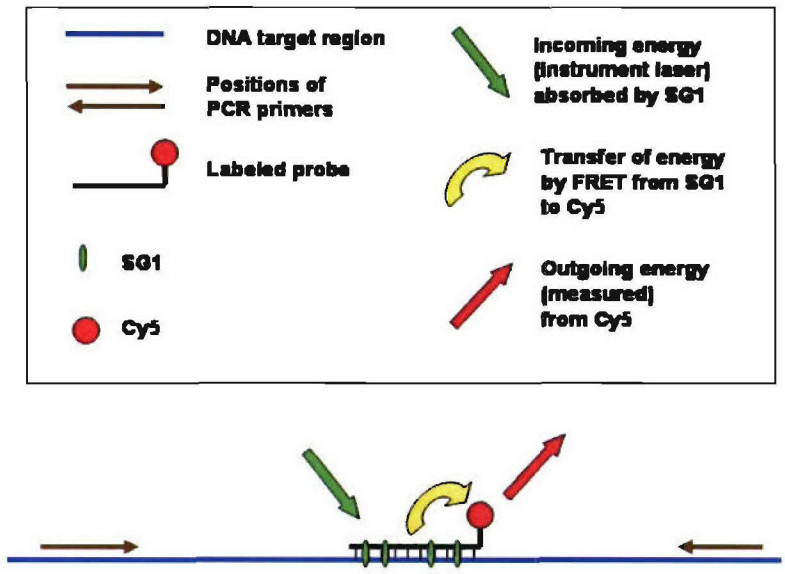

Figure 3 The variation used by Walker et al. (2001). SG1 (SybrGreen I) functions as FP-1, and Cy5 as FP-2. See text for further explanation.

Figure 4 shows the variation used by Lindler et al. (2001). In this case, probe 1 is labelled with Fluorescein (FL), which functions as FP-1, and probe 2 carries Red640, which functions as FP-2. FRET occurs when both probes bind to the target DNA sequence. When more target DNA is being amplified, more probe molecules bind to the target region, and more signal is measured.

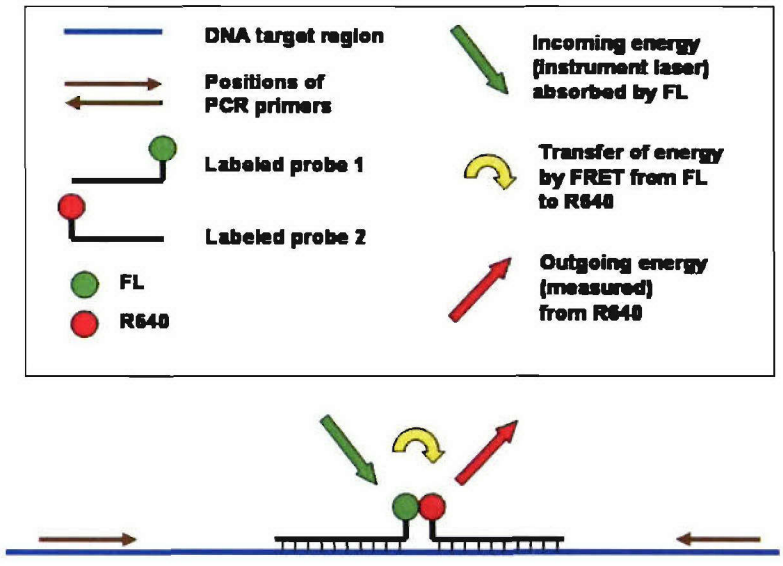

Figure 4 The variation used by Lindler et al. (2001). FL (Fluorescein) functions as FP-1, and (R640) Red640 as FP-2. See text for further explanation.

3.1.3 *Detection of mutations by measuring the melting point of attached probes*

As explained sofar, the two variations (according to Walker and Lindler, respectively) only measure the amplification of the target DNA sequence. This is the basic principle of real-time PCR. The practical use for measuring resistance lies in the fact that, after the amplification is completed, mutations can be detected by performing a so-called melting curve analysis (Broekhuijsen and Boomaars, 2001). Directly after amplification, the temperature of the reaction mix is slowly and continuously increased, e.g. at 0.05-0.1 °C per second. At the beginning of this stage, the maximum amount of probe molecules are bound to the target DNA, and a maximum signal is measured. At a certain temperature, one or both probe(s) will dissociate from the target DNA. When this occurs, FRET is no longer possible, and the signal drops dramatically. The temperature at which this occurs is called the dissociation temperature or melting point. The melting point is dependent on the binding energy of the probe(s), and consequently dependent on the percentage of the GC-content of the probe sequence (Broekhuijsen and Boomaars, 2001).

When a mutation is present in the region where the probe binds, the binding energy is much lower, because of the mismatch between the two DNA strands at the point of mutation. In this case, the melting point will be much lower. The presence of a mutation in the probe-binding region can thus be detected by measuring the melting point. Figure 5 illustrates this principle.

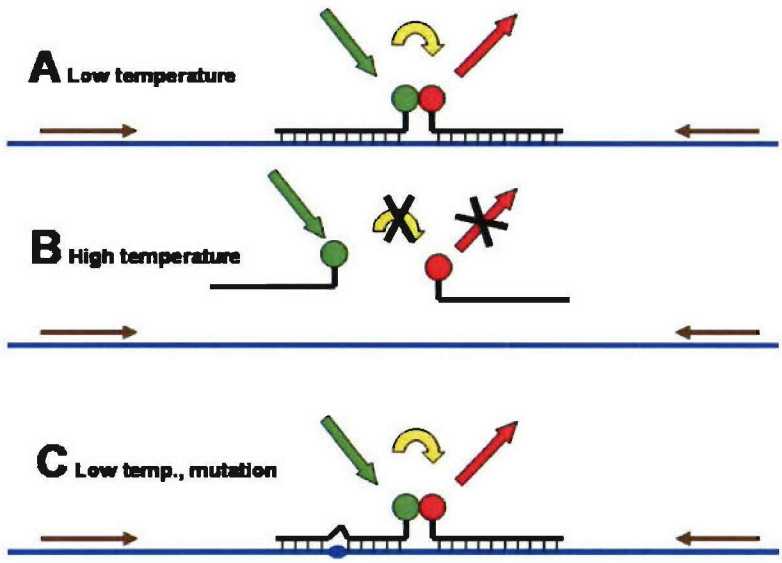

Figure 5 Detecting mutations by using melting point analysis. After amplification, at relatively low temperature, the probes are bound to the target DNA sequence (A). When the temperature is increased, the probes will dissociate at a specific temperature, the melting point (B). When a mutation is present (C), the melting point will be significantly lower.

3.1.4 *Experimental evaluation of two published methods*

Both methods (Walker et al. and Lindler et al.) were experimentally evaluated. Simply repeating their analyses was not possible, because the resistant strains they used (*Salmonella enterica* and *Yersinia pestis*, respectively) were not available. Therefore, both methods were adapted for use on a ciprofloxacin resistant strain of *Serratia marcescens*, described before (Broekhuijsen and van Dijk, 2005). This also gave the

advantage of comparing both methods with the same strain. For this purpose, the DNA sequences of the probes used by Walker et al. and Lindler et al. were modified to fit the sequence of the *gyrA* gene of *S. marcescens*.

The resistant strain of *S. marcescens* was known to contain a mutation at amino acid position 83 (Serine to Arginine) of the *gyrA* gene (Broekhuijsen and van Dijk, 2005). However, positions 81 and 87 are also known to be potentially linked with ciprofloxacin resistance. Therefore, probes should preferably cover all three potential mutation sites. The probe described by Walker et al. only covers positions 83 and 87, not 81, in the equivalent region of the *gyrA* gene of *S. enterica*. Lindler et al. use two probes, which together cover all three positions in the *gyrA* gene of *Y. pestis*.

Because the *gyrA* gene is conserved in evolution, it was not difficult to design the equivalent probes for the *gyrA* gene of *S. marcescens*.

```
S.ent GGTAAATACCATCCCCACGGCGATTCCGCAGTGTATGACACCATCGTTCGTATGGCGCAGCCATTCTCGCTG
Y.pes GGTAAATACCACCCGCATGGTGACAGCGCGGTCTACGACACTATCGTGCGTATGGCCCAGCCGTTCTCACTG
prot  G  K  Y  H  P  H  G  D  S  A  V  Y  D  T  I  V  R  M  A  Q  P  F  S  L
      75                81    83          87
S.mar GGTAAATATCACCCGCACGGTGACAGCGCGGTTTACGACACTATCGTGCGTATGGCTCAGCCGTTTTCACTG
Walker's method             ACAGCGCGGTTTACGACACT
Lindler's method    GCACGGTGACAGCGCGGTT ACGACACTATCGTGCGTATGGCTCA
```

Figure 6 Sequences of the target regions of the *gyrA* gene of *S. enterica* (top line), *Y. pestis* (second line), *S. marcescens* (fifth line). The differences in the DNA sequence in the first two species compared to *S. marcescens* are highlighted. The third line shows the protein sequence of *S. marcescens*, with positions 81, 83, and 87 highlighted (amino acid numbering in the fourth line). The two lower lines show the positions and sequences of the probes according to the two published methods, with the sequence adapted to *S. marcescens*.

Table 4 (Materials and methods) shows the designed probes for *S. marcescens* with all properties, including fluorescent labels. Probes GyrA-P01 and -P02 are according to Lindler, and GyrA-P03 is according to Walker. The sequences correspond to those in Figure 6. The sequence of probe GyrA-P03 is complementary to the one shown in Figure 6 and thus binds to the other DNA-strand in the *gyrA* gene.

The designed probes were tested according to the published methods. Primers for the PCR were used as described before (Broekhuijsen and van Dijk, 2005). Figure 7 shows the result obtained using the method of Walker et al. and Figure 8 shows the result obtained using the method of Lindler et al. Walker's method shows less decrease in melting point (6 degrees, Figure 7) of the resistant strain compared to the wildtype strain than Lindler's method (8 degrees, Figure 8). In addition, the melting peaks are much clearer with Lindler's method.

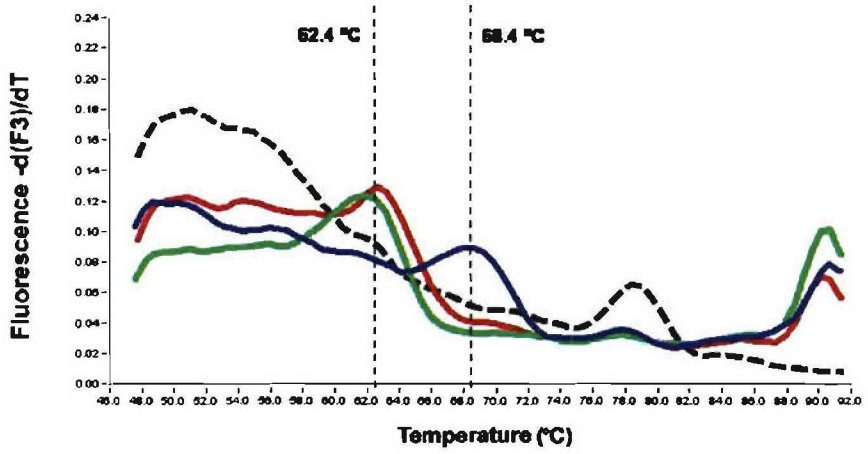

Figure 7 Melting point analysis of a wildtype and two ciprofloxacin resistant strains of *S. marcescens*, according to the method of Walker et al. The two resistant strains (red and green lines) show a melting point of 62.4 °C, 6 degrees lower than the wildtype (blue line).

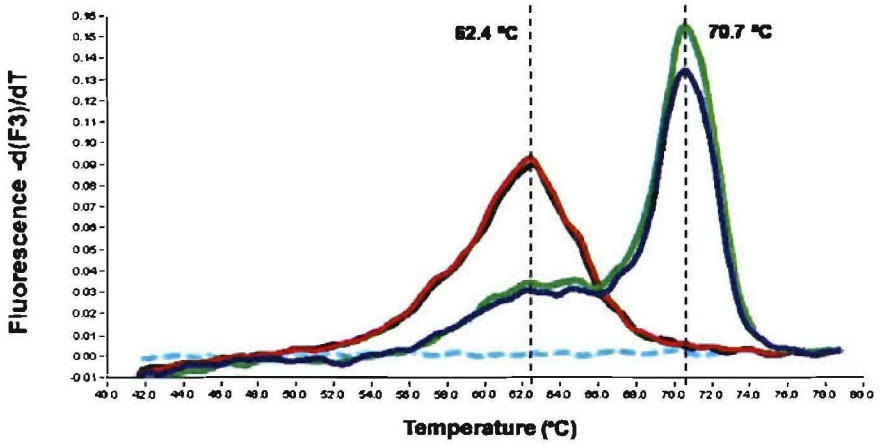

Figure 8 Melting point analysis of two wildtype and two ciprofloxacin resistant strains of S. marcescens, according to the method of Lindler et al. The two resistant strains (red and black lines) show a melting point of 8 degrees lower than the wildtype strains (green and blue lines).

3.2 Ciprofloxacin resistant strains of Bacillus anthracis

Sofar, Lindler's method for detection of point mutations in the *gyrA* gene seems effective for detecting ciprofloxacin resistance. However, this has only been published for Gram-negative strains, i.e. *Yersinia pestis* (by Lindler et al. 2001), *Salmonella enterica* (with a similar method, by Walker et al. 2001), and *Serratia marcescens* (this report).

As explained before (Broekhuijsen and van Dijk, 2005), the situation in Gram-positive strains might be different. Particularly, mutations that confer resistance to ciprofloxacin might be found in the *grlA* gene, either in addition to the *gyrA* gene or even preferentially.

Therefore, it was decided to induce ciprofloxacin resistance in a Gram-positive strain. For this purpose, it was decided to use *Bacillus anthracis*, which is the most relevant pathogen for the application. Since it is not desirable to render a dangerous pathogen such as *B. anthracis* more dangerous by inducing resistance, a non-virulent strain was used. Strain BM232 (ATCC4229) which lacks the virulence determining plasmid pXO1 seemed suitable for this. To ensure that no mix-up would occur, the identity of the strain and particularly the absence of the pXO1 plasmid was confirmed using real-time PCR analysis, with specific primers for pXO1, pXO2, and a chromosomal marker for *B. anthracis* (described in Broekhuijsen et al., 2004).

The result confirmed that the strain to be used for inducing resistance was *B. anthracis*, containing plasmid pXO2 but lacking plasmid pXO1. The same real-time PCR analysis was also done after induction of resistance, to ensure that all resistant strains still were non-virulent.

3.2.1 *Induction of ciprofloxacin resistance*

It was attempted to secure at least 10 isolates of each level of resistance against ciprofloxacin, starting at 0.5 µg/ml and doubling up to 64 µg/ml. However, it was difficult to obtain isolates with the highest levels of resistance. At levels of 32 and 64 µg/ml ciprofloxacin, only 6 and 3 independent colonies were secured, respectively (Table 5). In addition, these isolates with high resistance showed poorer growth than normal.

Table 5 Overview of resistant isolates from a non-virulent strain of *B. anthracis*.

Level of resistance (µg/ml cipro)	Number of isolate secured	Isolate designations	Comment
0.5	10	BM232-02 through -11	Low resistance level
1	10	BM232-12 through -21	Low resistance level
2	10	BM232-22 through -31	Low resistance level
4	10	BM232-32 through -41	
8	10	BM232-42 through -51	
16	10	BM232-52 through -61	
32	6	BM232-62 through -67	Poor growth
64	3	BM232-68 through -70	Poor growth

3.2.2 *DNA sequencing of resistant strains*

A selection of resistant strains were further analysed. In particular, the sequences of the DNA regions called Quinolone Resistance Determining Region (QRDR, Broekhuijsen and van Dijk, 2005) of three related genes were of interest. PCR primers were designed to amplify each region, i.e. the QRDR of the *gyrA*, *grlA*, and *grlB* genes of *B. anthracis*.

Not all sequencing reactions or amplifications were successful, and no data were obtained from strains with resistance levels of 2, 8, and 32 µg/ml. However, the overall result shows a clear pattern. At all levels of resistance, including 0.5 µg/ml, the same point mutation was found, i.e. C to T at position 254 of the *gyrA* gene (Serine to Leucine at position 85 in the amino acid sequence). Up to a resistance level of 16 µg/ml no mutations were found in the *grlA* and *grlB* genes. At the highest level (64 µg/ml) an additional mutation was found, i.e. C to T at position 242 of the *grlA* gene (Serine to Phenylalanine at position 81 of the amino acid sequence). It is uncertain if this additional mutation also occurs at 32 µg/ml, because of lack of data with these strains. At 64 µg/ml, no mutations were found in the *grlB* gene. Table 6 summarizes the results.

Table 6 Overview of mutations found in resistant strains of *B. anthracis*. n.d.: no data.

Mutant strain	Resistance (µg/ml)	*gyrA* nucleotide	*gyrA* protein	*grlA* nucleotide	*grlA* protein	*grlB*
BM232 (orig. strain)	0	no mutation	no mutation	no mutation	no mutation	no mutation
BM232-02	0.5	C254 to T	S85 to L	no mutation	no mutation	no mutation
BM232-07	0.5	C254 to T	S85 to L	no mutation	no mutation	no mutation
BM232-32	4	C254 to T	S85 to L	no mutation	no mutation	no mutation
BM232-33	4	C254 to T	S85 to L	no mutation	no mutation	no mutation
BM232-57	16	C254 to T	S85 to L	no mutation	no mutation	n.d.
BM232-58	16	C254 to T	S85 to L	no mutation	no mutation	n.d.
BM232-59	16	n.d.	n.d.	no mutation	no mutation	no mutation
BM232-60	16	n.d.	n.d.	n.d.	n.d.	no mutation
BM232-68	64	C254 to T	S85 to L	C242 to T	S81 to F	no mutation
BM232-69	64	C254 to T	S85 to L	C242 to T	S81 to F	no mutation

3.3 Use of Lindler's method on Bacillus anthracis

The ciprofloxacin-resistant strains of *B. anthracis* were used to further test Lindler's method. Since mutations related to ciprofloxacin-resistance in *B. anthracis* were found in two genes (*gyrA* and *grlA*), as predicted from published data, Lindler's method was adapted for both genes.

Sequences for primers and probes were adapted to the sequence of the two genes of *B. anthracis* (data see Materials and methods). The protocol for the real-time PCR was the same as before. For the *gyrA* gene, a similar result was obtained as with *S. marcescens*, with a 3 °C difference between the melting peak of the original strain of *B. anthracis* and 8 resistant strains, with resistance levels of 0.5, 1, 2, 4, 8, 16, 32, and 64 µg/ml (Figure 9).

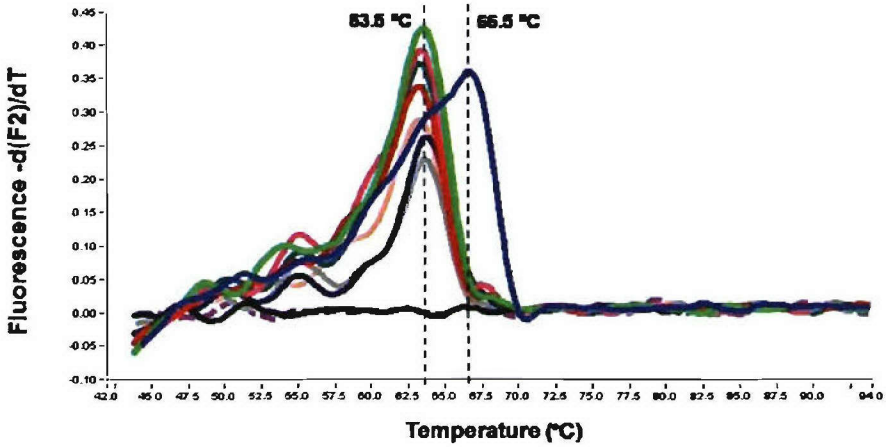

Figure 9 Melting point analysis of the original *B. anthracis* strain and 8 ciprofloxacin resistant strains, according to the method of Lindler et al., adapted for the *gyrA* gene. The 8 resistant strains (with resistance levels of 0.5, 1, 2, 4, 8, 16, 32, and 64 µg/ml) show a melting point of 63.5 °C, 3 degrees lower than the original strain (blue line).

For the *grlA* gene, the result was also similar, with a 2.5 °C difference in temperature of the melting peak (Figure 10). However, in this case only the high-level resistant strains showed a melting peak with lower temperature, completely in accordance with the

mutations that were found in the DNA sequence (see above). The strains that were used for Figure 10 had resistance levels of 0.5 µg/ml (2 strains), 8 µg/ml (2 strains), 16 µg/ml (2 strains), 32 µg/ml (2 strains), and 64 µg/ml (3 strains). Only the 3 strains with a resistance level of 64 µg/ml had a lower melting point (Figure 10).

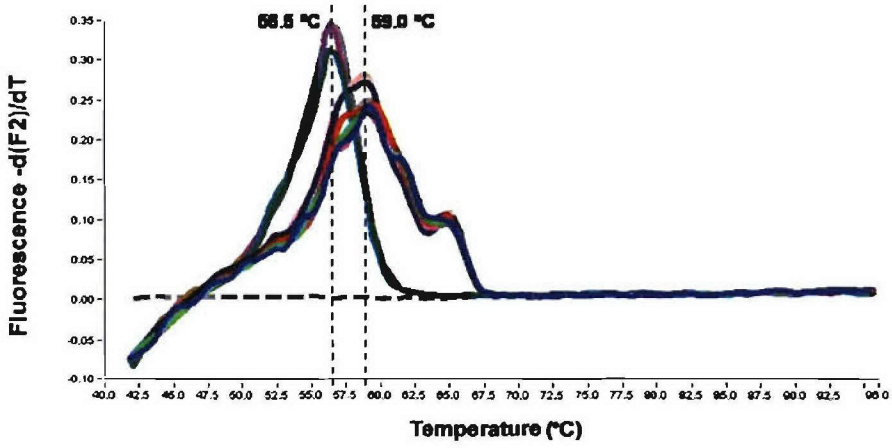

Figure 10 Melting point analysis of the original *B. anthracis* strain and 11 ciprofloxacin resistant strains, according to the method of Lindler et al., adapted for the *grlA* gene. Eleven resistant strains (with resistance levels of 0.5, 8, 16, 32, and 64 µg/ml) were analysed. Only the 3 strains with a resistance level of 64 µg/ml show a melting point of 56.5 °C, 2.5 degrees lower than the original strain (blue line).

3.4 Analysis of MRSA strains

Seven Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were obtained from RIVM. These had been characterised by RIVM as MRSA using classical test methods including culturing and commonly used screening methods for determining resistance levels to a range of antibiotics. Most strains were resistant against multiple antibiotics. According to the data supplied by RIVM, six of the seven strains were also resistant to ciprofloxacin, with only strain BM292 sensitive to ciprofloxacin. Strain BM187, which is a non-MRSA strain of *Staphylococcus aureus*, was also analysed, as a control.

All eight strains (BM187 and BM292-298) were analysed using the commercial kit for LightCycler detection of MRSA from Roche Diagnostics. The results were not completely in agreement with expectations. The positive control (supplied with the kit) was positive, water as negative control was negative, and strain BM187 (non-MRSA) was negative. Six out of the seven MRSA strains were positive, but one was negative (Figure 11). This was strain BM292, which was also sensitive to ciprofloxacin.

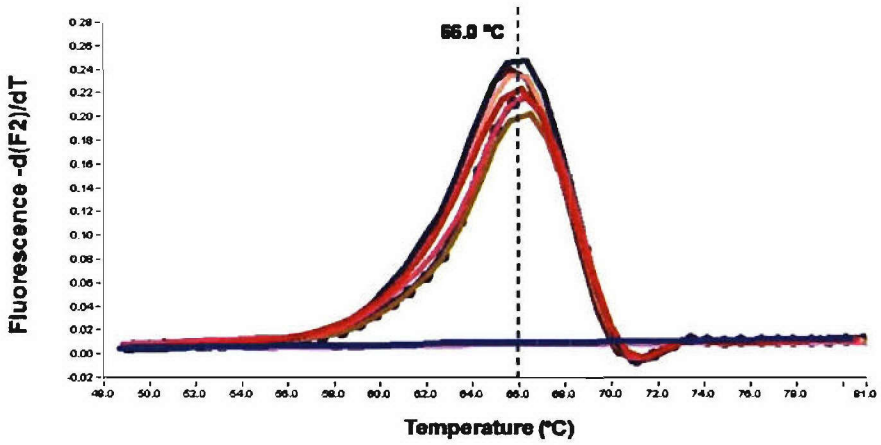

Figure 11 Melting point analysis using the MRSA detection kit (Roche Diagnostics) on 8 strains of *S. aureus*. The negative control, the non-MRSA strain, and one MRSA strain are negative. All other six MRSA strains, as well as the positive control from the kit, are positive, showing a melting peak at 66 °C.

Next, two genes associated with ciprofloxacin resistance, i.e. the *gyrA* gene and the *grlA* gene, were analysed for the presence of mutations. Primers designed for the *gyrA* gene (GyrA-F16/-R16) and *grlA* gene (GrlA-F01/-R01) of *S. aureus* were used to prepare amplicons of the QRDR regions of both genes. The DNA sequences of purified amplicons were determined and compared to the wildtype sequence of the genes.

The results are summarised in Table 7. No mutations were found in the control strain BM187 and the MRSA strain BM292 (both cipro-sensitive), nor in the MRSA strain BM297 (cipro-resistant). All other cipro-resistant MRSA strains (BM293, -294, -295, -296, -298) were found to have mutations in both genes, at position 84 in the *gyrA* gene and position 80 in the *grlA* gene. Interestingly, strain BM294 had a different mutation in the *gyrA* gene (S84-A instead of S84-L) compared to the other strains.

Table 7 Summary of mutations in the DNA in the *gyrA* and *grlA* genes.

Strain	Mutation in *gyrA* gene	Mutation in *grlA* gene	Cipro-resistance (µg/ml)	Comment
BM292	no mutation	no mutation	1	cipro-sensitive
BM293	S84 to L	S80 to F	9	
BM294	S84 to A	S80 to F	8	
BM295	S84 to L	S80 to F	9	
BM296	S84 to L	S80 to F	9	
BM297	no mutation	no mutation	9	
BM298	S84 to L	S80 to F	8	
BM187	no mutation	no mutation	0	non-MRSA, cipro-sensitive

4 Discussion and conclusions

Firstly, all results are discussed in the same order of paragraphs in Chapter 3, as follows:

1 Evaluation of two published methods for detecting ciprofloxacin resistance (paragraph 4.1).
2 Induction of ciprofloxacin resistance in a non-virulent strain of *Bacillus anthracis* and DNA sequence analysis of obtained ciprofloxacin resistant (mutant) strains (paragraph 4.2).
3 Use of Lindler's method on *Bacillus anthracis* (paragraph 4.3).
4 Analysis of MRSA strains (paragraph 4.4).

Secondly, operational issues are discussed (paragraphs 4.5) and a summary of conclusions is given (paragraph 4.6).

4.1 Published methods

4.1.1 Lindler and Walker

The publication by Walker et al. (2001) describes the use of real-time PCR for fast detection of mutations in the *gyrA* gene of *Salmonella enterica*. The mutations that are detected are connected to ciprofloxacin-resistance. Lindler et al. (2001) do the same with *Yersinia pestis*. These two publications are not the only ones (see below), but they are among the first, and they describe two variations that can be used as alternative approaches. Therefore these two methods seemed attractive to evaluate in this study.

To evaluate the two methods, it was necessary to use a pair of sensitive and resistant strains of the same species, and with known mutations in the resistant strain. The only suitable pair of strains available at the time of evaluation was the wildtype and resistant strains of *Serratia marcescens* described earlier (Broekhuijsen and van Dijk, 2005). In addition, using this pair of strains for both methods would ensure an equal comparison between the two methods.

Using the *S. marcescens* strains clearly showed that Lindler's method gives better results, i.e. a clear distinction between the two strains, with strong melting peaks. Lindler's method also has a theoretical advantage over Walker's method, because Lindler et al. use two probes (Walker et al. use one probe), which covers a larger region of the gene, and is therefore more likely to cover all possible mutations related to ciprofloxacin resistance. As shown above, Walker's single probe does not cover amino acid 81, where mutations are known to occur, whereas Lindler's two probes cover all the important positions (amino acids 81, 83, and 87). Therefore, Lindler's methods seemed theoretically to be better. It also gave better results. Lindler's method was therefore selected as method of choice.

4.1.2 Additional published methods

Others have thought of this method as well, which is not surprising. The LightCycler equipment is, amongst other applications, often used with success for fast and reliable mutation analysis. Below are some examples of publications describing similar methods. They were not evaluated in this study, because they did not appear to have additional value over Lindler's or Walker's method.

Wilson et al. (2000), in probably the first publication describing such a method, use a similar method on ciprofloxacin resistant *Campylobacter jejuni*. According to this publication, high-level resistance to ciprofloxacin (>= 16 µg/ml) is predominantly associated with a mutation in codon 86 (C to T) of the *gyrA* gene. The method uses the so-called Taqman system (Broekhuijsen and Boomaars, 2001). It appears to be not as straightforward as the method with the LightCycler mutation assay. The targeted region of the *gyrA* gene covers codons (amino acids) 82-90 of the *gyrA* gene.

Carattoli et al. (2002) described a similar *gyrA* mutation assay for identification of ciprofloxacin resistant *Campylobacter coli*. They target codon 86 of the *gyrA* gene, using clinical strains that were isolated in Italy in 2000. They use the same method as Lindler's, with two probes, and melting peak analysis on the LightCycler.

Li et al. (2002) described a method for rapid detection of quinolone resistance-associated *gyrA* mutations in *Neisseria gonorrhoeae*. They also use the LightCycler mutation assay and target codons 91 and 95 of the *gyrA* gene. They also state that the mutation status in the *gyrA* gene (as assessed by the LightCycler assay) was completely in agreement with the results of their previous conventional sequencing analysis of ciprofloxacin resistant strains.

All these published studies describe methods using Gram-negative bacteria and target the same region of the *gyrA* gene, i.e. codons (amino acids) 81-87 for most strains, or codons 91-95 for some other strains (e.g. *Neisseria*). This short overview of published method shows three things:

1 The method is suitable for a range of Gram-negative bacteria, possibly all.
2 Analysis with the LightCycler mutation assay completely correlates with genomic mutations found by DNA sequence analysis.
3 Ciprofloxacin resistance in naturally isolated Gram-negative strains is very likely associated with mutations in a small region of the *gyrA* gene.

Although resistant strains with mutations in other genes have been described, the overwhelming majority of Gram-negative resistant strains seems to have mutations in the targeted region of the *gyrA* gene.

In Gram-positive strains, the situation seems to be slightly different (see below).

4.2 Induction of resistance and analysis of mutants

Because of difficulties in obtaining resistant strains from other sources (e.g. hospitals), induction of resistance was necessary to evaluate the method. Careful experimentation and thorough screening is needed for this, but otherwise it is not very difficult to achieve resistance levels of up to 8 µg/ml ciprofloxacin, or even higher.

Analysis of mutants that were obtained in this study showed results that are in agreement with published data:

- Gram-negative strains with a moderate or high resistance level (e.g. at or above 8 µg/ml) have at least one mutation in the region 81-87 of the *gyrA* gene. *Neisseria* and possibly some other species might form an exception, with mutations in the region 91-95 (see above).

- Gram-positive strains show mutations in the same region of the *gyrA* gene at much lower levels of resistance (e.g. 1 µg/ml), and at higher levels additional mutations are found in the *grlA* gene.

The consequence of these findings is that for Gram-negative strains a single assay for the *gyrA* gene is sufficient, whereas for Gram-positive strains two assays are needed, one for the *gyrA* gene and one for the *grlA* gene.

4.3 Use of Lindler´s method on Bacillus anthracis

Lindler's method was successfully adapted for *Bacillus anthracis*. Resistant strains with known mutations all gave the expected results, both for the *gyrA* gene and the *grlA* gene. The difference in melting peak for the *gyrA* and *grlA* genes of *B. anthracis* (2.5-3.0 °C) was not as large as for the *gyrA* gene of *S. marcescens* (8.0 °C), but in all cases the difference between the original strain and the mutant strains is clearly visible, which makes the method suitable.

An important observation is that, without exception, all mutations that were found with DNA sequence analysis, were also reflected in a lower melting peak with the LightCycler mutation assay.

Using Lindler's method with success on the *gyrA* gene of *Serratia marcescens* (Gram-negative) and on the *gyrA* gene and *grlA* gene of *Bacillus anthracis* (Gram-positive) indicates that this method can be applied to a wide range of species, and presumably on all bacterial species.

4.4 Analysis of MRSA strains

MRSA strains are not considered biowarfare agents. However, they are widespread in the whole world, and should especially be taken seriously when people are hospitalised outside North-Western Europe and North America. This also applies to military personnel during out of area operations. Infection with MRSA confronts the person involved with a nasty problem, especially when the patient is already (seriously) injured or ill.

The set of MRSA strains was useful to analyse for two reasons:

1. A commercial kit is available for detection of MRSA that uses the same instrument (LightCycler) and technology (real-time PCR in combination with melting peak analysis) as Lindler's method for detecting ciprofloxacin resistance. It is therefore easily combined.
2. Most of the MRSA strains in the set (6 out of 7) were also known to be resistant to ciprofloxacin. It was therefore logical to see whether these strains contained mutations at the expected positions in the expected genes.

The commercial kit for detection of MRSA strains targets the mecA gene, which is associated with methicillin resistance. The MRSA kit showed clear results. However, one MRSA strain (BM292) was not detected as such by the kit. This gave doubt whether BM292 is really an MRSA strain, particularly because it is also the only strain of the set of MRSA strains that lacks ciprofloxacin resistance (and the only one that lacks resistance to several other antibiotics, data not shown). The data obtained with the MRSA kit were presented to RIVM (supplier of the MRSA strains), but they could not

confirm whether BM292 is MRSA or not. At present, it is more likely that the kit gave the correct result, and consequently that BM292 is not MRSA.

As with the mutation assay, the analysis with the MRSA kit takes about 90 minutes to perform, including set-up and interpretation.

DNA sequence analysis of the 7 MRSA strains (or 6, if not counting BM292) revealed mutations in the expected genes, at the expected positions. These are codon 84 in the *gyrA* gene and codon 80 in the *grlA* gene. Two interesting details were found:

1 One resistant strain (BM294) had a slightly different mutation in the *gyrA* gene, although at the same position.
2 One resistant strain (BM297) did not show mutations in the analysed regions of the *gyrA* and *grlA* genes. Obviously, resistance in this strain is caused by (an)other mutation(s). As stated before (Broekhuijsen and van Dijk, 2005), the absence of mutations in the QRDR regions of *gyrA* and *grlA* does not always exclude the possibility of resistance. This is important to realise, and is clearly demonstrated with this set of clinical MRSA strains.

LightCycler mutation assays for the *gyrA* and *grlA* genes of *S. aureus*, according to Lindler were not designed during this study, but will be in the future.

4.5 Operational issues

4.5.1 Practical use of the method

In a typical operational setting where a bio threat is detected or suspected, one would first aim at identifying the species of a potential pathogenic organism. This is often done using real-time PCR, e.g. with the LightCycler instrument. If a bacterial species from the threat list (or other relevant species) is identified, the LightCycler mutation assay for detecting ciprofloxacin resistance could immediately be started, using the same equipment and type of reagents. Use of the same platform (LightCycler) and similar technology (real-time PCR with melting peak analysis) for both identification of species and detection of ciprofloxacin resistance, is obviously a great advantage.

Reagents for detecting ciprofloxacin resistance should consist of the standard real-time PCR reagents, together with specific primers and probes for the targeted organisms. It has been demonstrated that PCR reagents can be pre-mixed and freeze-dried, which greatly simplifies analysis outside laboratories. As with all assays, incorporated controls are important, and protocols should be clear and concise.

For Gram-negative species, a *gyrA* gene mutation assay would suffice, whereas for Gram-positive species, a *gyrA* gene and *grlA* gene mutation assay would be necessary. Mutation assays are specific for each species, and consequently a set of reagents for all relevant species should be available.

4.5.2 Time scale of method

The time required for detecting ciprofloxacin resistance in a strain can be roughly estimated.

Set-up of reaction	15 minutes
Reaction run	60 minutes
Interpretation	15 minutes
Total time required	90 minutes

The time required can be less than shown here, depending on the training level of operators.

4.5.3 Success rate of method

As demonstrated above, ciprofloxacin resistance is mostly associated with mutations at specific positions in the QRDR region of the *gyrA* gene, in Gram-negative bacteria, and of the *gyrA* and *grlA* genes in Gram-positive bacteria.

However, exceptions occur, and this means that finding a wildtype sequence in the QRDR region of these genes does not guarantee that the strain analysed is susceptible to ciprofloxacin. This is clearly illustrated with one of the MRSA strains used in this study. Both the results of this study, and data from numerous publications, show that in the majority of cases resistance does correlate with mutations in the targeted DNA regions. The usefulness of this method lies in the fact that in most cases, resistant strains will be easily recognized, and much faster than with other methods.

Not enough data exist to reliably estimate the success rate (the percentage of cases where resistance is caused by a mutation at the expected positions), but 80%-90% would be a conservative estimate. In practice, it could be much higher.

4.6 Summary of conclusions

The most relevant statements and conclusions are summarized below.

- It is difficult to obtain ciprofloxacin resistant strains of the relevant species for testing of the method.
- Experimental induction of ciprofloxacin resistance is possible, as shown with *B. anthracis* (and with *S. marcescens*, in an earlier report).
- Mutations in the expected regions of the genes associated with resistance were found in all cases, except in one MRSA strain.
- Resistant Gram-negative strains show mutations in the *gyrA* gene. Resistant Gram-positive strains show mutations in the *gyrA* gene and *grlA* gene. Presence of additional mutations can depend on the level of resistance.
- The presence of a mutation always correlated with a shift in melting point in a LightCycler mutation assay, meaning that the mutation assay correctly reveals DNA mutations.
- The success rate of the method is thought to be 80%-90% or more.
- The time scale of the method is estimated to be 90 minutes.

5 Further research and recommendations

5.1 Further research

Further research can be based on the results described in this report. Lindler's method is shown to be effective, and can be extended to a list of relevant species. It should be designed for the *gyrA* gene for all relevant Gram-negative species, and for both the *gyrA* gene and the *grlA* gene for Gram-positive species.

Adaptation of the method for the *grlB* gene can be considered, but this should be preceded by DNA sequence analysis of the *grlB* gene from highly resistant strains. It should only be done if mutations are found in the *grlB* gene.

Resistant strains remain essential for validating the method. It would be desirable to have (or to induce) more resistant strains, of different species.

Resistance against other antibiotics and/or resistance in other pathogens can be considered as important topics for further research.

5.2 Recommendations

The following recommendations are made.

- To continuously search for and obtain (either acquire from elsewhere or induce experimentally) additional resistant strains, for further validation of the method.
- To extend the number of PCR primers and probes, for all relevant genes, for all relevant species.
- To seek for exchange of information and data on this topic with other parties, e.g. foreign defence research.
- If the method is considered to be implemented for operational use, to design ready-to-use reagent mixtures, and to test the performance and stability of these.

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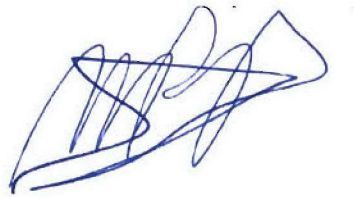

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Fast DNA-based methods for detecting antibiotic resistance in bacteria were evaluated. The best method was adapted for use with several species, including *Bacillus anthracis*. The method was shown to be widely applicable, reliable, and performed within 90 minutes. DNA mutations associated with ciprofloxacin resistance were examined, and can be used to extend the method to other bacterial species.

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