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(54) Title: ANTIBIOTIC SUSCEPTIBILITY AND VIRULENCE FACTOR DETECTION IN PSEUDOMONAS AERUGINOSA

(57) Abstract: The present invention relates in general to the detection of antibiotic resistance determinants in Pseudomonas aeruginosa (P. aeruginosa). The present invention discloses a micro-array for the detection of antibiotic resistance determinants and mutations in said organism, a method for the detection of said determinants and a kit. This micro-array concept offers the rapid sensitive and specific identification of antibiotic resistance profiles.

Antibiotic susceptibility and virulence factor detection in Pseudomonas aeruginosa

Field of the invention

The present invention relates in general to the detection of Pseudomonas aeruginosa (P. aeruginosa) strains exhibiting multi-resistance to antibiotics. In particular, the present invention pertains to a micro-array for the detection of antibiotic resistance determinants in said organism, a method for the detection of said determinants and a kit. This micro-array concept offers the rapid, sensitive and specific identification of antibiotic resistance profiles. It is easily expandable and may thus be adapted to changed clinical and epidemiological requirements in clinical diagnosis as well as in epidemiological studies.

State of the art

P. aeruginosa is an opportunistic pathogen associated with nosocomial infections of immunocompromised patients especially in intensive care units (ICUs). P. aeruginosa is responsible for approximately 10% of all infections on ICUs and results in a high mortality and morbidity when associated with pneumonia or septicemia (Prevention, C.f.D.C.a.; Am. J. Infect. Control. 24 (1996), 380–388). This organism is characterized by an intrinsic resistance to various antimicrobial agents and an ability to develop multiresistance during antibiotic therapy (Livermore, D.M.; Clinical Infectious Diseases 34 (2002), 634–40). The intrinsic multiresistance results from the synergy between broadly specific drug efflux pumps and a low degree of outer membrane permeability. A variety of efflux systems have been identified to date, including the well characterized MexAB- OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM efflux pumps (Masuda, N., et al.; Antimicrob. Agents Chemother. 44(12) (2000), 3322-7). MexAB-OprM is constitutively expressed in wild type P.aeruginosa PAO1, whereas the other efflux systems are not. Mutations in regulatory genes of these efflux

systems (mexR, mexT, nfxB) can either cause overexpression of MexAB-OprM or may induce expression of the other regulated efflux systems. P. aeruginosa may as well harbour different plasmid encoded antibotic resistance genes like -lactamases (tem, shv, oxa), aminoglycoside modifying enzymes (aac, aad, aph) and carbapenemases (imp, vim). These plasmids can be easily acquired via horizontal gene transfer from other gram-negative organisms, especially in a clinical setting.

So far, detection of P. aeruginosa has been performed by isolating nucleic acid sequences from clinical samples and analyzing them by either using gel electrophoresis of DNA fragments (e.g. of restriction fragments) – the so-called southern blot, hybridization events, and the direct sequencing of DNA (for example according to the Maxam-Gilbert method). All of the above-mentioned methods are widely used in biological sciences, medicine and agriculture. The deficiencies of the three methods reside, however, in that even though southern blots and hybridization experiments may be carried out relatively fast, they are only useful for the analysis of short DNA strands. The DNA sequencing results in the accurate determination of the nucleic acid sequences, but is time consuming, expensive and connected with certain efforts when applied to greater projects, e.g. the sequencing of a complete genome.

Known methods to detect the presence of P. aeruginosa in a clinical sample reside e.g. in real-time polymerase chain reactions (cf. US-2004/248148) or other PCR based assays (cf. US-2003/180733), which use primers specific for particular genes of said organism. Also, the use of enzyme linked immunosorbant assays and Western blot immunoassays for the detection of P. aeruginosa is disclosed for example in US-6,551,795 and EP-0 265 672.

Since these phenotypic based microbiological and biochemical techniques for species identification and antibiotic susceptibility determination require at least two days, a reliable therapy is not possible in urgent cases of critical ill patients. The development of new and faster methods is therefore a crucial point to allow a better adjustment of the antibiotic treatment of severe infections caused by multiresistant pathogens.

The micro-array technology represents in contrast to e.g. PCR and antibody basing methods, a tool for a highly specific, parallel detection of thousands of different DNA sequences in a single experiment (Schena, M. et al.; Science 270 (1995), 467-470). Micro-arrays which are in some cases also referred to as hybridization arrays, gene arrays or gene chips comprise in brief a carrier or support on which at defined locations at a possibly high density capture molecules are attached directly or via a suitable spacer molecule. The spacer molecules may be considered to function as a "bridge" between the capture molecule and the surface of the carrier to allow an easier attachment of the capture molecule. Said capture molecules consist of relatively short nucleic acid sequences, in particular DNA, which is capable to hybridize specific to the target molecules or probe molecules to be analyzed resulting usually in DNA:DNA or DNA:RNA hybrids. The occurrence of the hybridization event is than detected with for example fluorescent dyes and analyzed.

The advantages of the micro-array concept resides preliminary in its ability to carry out very large numbers of hybridization-based analyses simultaneously. Originally developed for the analysis of mammalian gene expression, an increasing number of reports on micro-arrays for identification and characterization of prokaryotes also used in microbial diagnostics was encountered in recent years (Bodrossy, L. and A. Sessitsch; Curr. Opin. Microbiol. 7 (2004), 245-254). Combination of PCR based pre-amplification steps with subsequent micro-array based detection of amplicons on a micro-array facilitates the sensitive and highly specific detection of PCR products (Call, D. R. et al.; Int. J. Food Microbiol. 67 (2001), 71-80). Amplicons are identified by a specific hybridization reaction on the array thus reducing the risk of wrong positive results due to the occurrence of nonspecific bands after PCR. Besides that, micro-arrays utilizing oligonucleotides as capture probes enable the detection of single nucleotide polymorphisms (SNPs) such as resistance mutations without the need for additional sequencing. However, only a few studies describe the development of diagnostic micro-arrays for the molecular detection of bacterial antibiotic resistance, targeting either a limited number of acquired antibiotic resistance genes or resistance mutations in various genes.

The use of micro-arrays for the detection of pathogenic bacteria is for example disclosed in

WO 03/031654, wherein a micro-array with probes for genotyping Mycobacteria species, differentiating Mycobacterium strains and detecting antibiotic-resistant strains is specified. The simultaneous performance on multiple clinical isolates through a single test of a Mycobacterium genotyping test, M. tuberculosis strain differentiation test and an antibiotic-resistance detection test is specified.

WO 01/7737 relates to the identification (detection and/or quantification) of (micro-) organisms among others having homologous nucleotide sequences by identification of their nucleotide sequences, after amplification by a single primer pair. Organisms of the same genus or family may and/or related genes in a specific (micro) organism present in a biological sample may be identified or quantified.

Methods for assaying drug resistance and kits for performing such assays are disclosed in the US-6,013,435. Target sequences associated with genetic elements are selectively amplified and detected. The methods described herein are especially useful for screening of Microorganisms, which are difficult to culture.

In US-2003143591 methods and strategies to detect and/or quantify nucleic acid analytes in micro-array applications such as genotyping (SNP analysis) are disclosed. Nucleic acid probes with covalently conjugated dyes are attached either to adjacent nucleotides or at the same nucleotide of the probe while novel linker molecules attach the dyes to the probes.

The disadvantages of the techniques according to the state of the art for the detection of P. aeruginosa reside in that they require long runs and are solely adaptive to a limited number of samples to be tested and often also expensive. Additionally, no method is known which uses simultaneously several nucleic acids probe for the detection of multiple antibiotic resistance determinants and optionally other virulence factors to facilitate an overview on the resistance properties of a single strain and gives valuable and sometimes life-saving information about a suitable treatment.

Object of the invention

The present invention provides a micro-array as a genotype based method for detecting antibiotic susceptibility of P. aeruginosa, which incorporates nucleic acids for targeting determinants of multi-resistant P. aeruginosa and optionally specific controls. The micro-array enables a rapid, accurate and inexpensive identification of antibiotic resistance profiles of P. aeruginosa. The inclusion of nucleic acids representing virulence factors, like toxins or alginate, broadens the information about the virulence potential of P. aeruginosa at the same time. Said micro-array is easily expandable and may thus be adapted to changing clinical and epidemiological requirements in clinical diagnosis as well as in epidemiological studies. A fast and reliable assay with a high throughput may be helpful in reducing the spread of multiresistant isolates and improves the treatment options of severe and often life-threatening P. aeruginosa infections.

In the figures

In Fig. 1, an embodiment of a micro-array according to the invention is shown. All capture probes were spotted in triplicates. The mutation position is assigned for single nucleotide polymorphisms (SNPs) and the insertions and deletions of respective genes. Modifying enzyme genes are named according to their substrate specificity. Genes relevant for resistance by their presence were named with the usual name. The different genes are indicated in the array legend. For SNPs, the central base in the probe A, T, G, C is spotted in one row below the other, for insertions and deletions, a wild-type probe below a mutation probe, and for gene presence an anti- sense down to sense probe.

Fig. 2 shows a genotype analysis of respective resistance and virulence genes of the clinical P. aeruginosa isolate No. 23 (b), which was performed using the inventive micro-array and were compared with wild-type P. aeruginosa PAO1 (a). The signal intensity is shown in false color, in intensity increasing from grey to white. The frames highlight the positions in which the two isolates differ from each other.

In Fig. 3, the percent of mismatch probes depending on the mismatch positions (MM)/perfect match position (PM) ratio from all hybridization experiments of the P. aeruginosa test

collective is shown.

Fig. 4 shows a genotype analysis by the present micro-array of 3 consecutive P. aeruginosa isolates collected from the same patient Array detail of the 3 isolates (No. 1 = a, No. 2 = b, No. 3 = c) covering gyrA and parC and aminoglycoside modifying enzymes is shown. The signal intensity is shown in false color, in increasing intensity from grey to white.

Definitions

The term "micro-array" as used herein refers to a carrier or support respectively, which is preferably solid and has a plurality of molecules bound to its surface at defined locations or localized areas. The molecules bound to the carrier comprise nucleic acid sequences, the capture molecules, which are specific for a given or desired target sequence. The sequences may be bound to the carrier via spacer molecules, which bind each capture nucleotide to the surface of the support. In the above context a localized area is an area of the carrier's surface, which contains capture molecules, preferably attached by means of spacers to the surface of the carrier, and which capture molecules are specific for a determined target/ probe molecule.

"Spacers" are molecules that are characterized in that they have a first end attached to the biological material and a second end attached to the solid carrier. Thus, the spacer molecule separates the solid carrier and the biological material, but is attached to both. The spacers may be synthesized directly on or may be attached as a whole to the solid carrier at the specific locations, whereby masks may be used at each step of the process. The synthesis comprises the addition of a new nucleotide on an elongating nucleic acid in order to obtain a desired sequence at a desired location by for example photolithographic technologies which are well known to the skilled person. Bindings within the spacer may include carbon-carbon single bonds, carbon-carbon double bonds, carbon-nitrogen single bonds, or carbon-oxygen single bonds. The spacer may be also designed to minimize template independent noise, which is the result of signal detection independent (in the absence) of the template. In addition, the spacer may have side chains or other substitutions. The active group may be reacted by suitable means to form for example preferably a covalent bound between the

spacer and solid carrier, capture or probe molecule. Suitable means comprise for example light. The reactive group may be optionally masked/protected initially by protecting groups. Among a wide variety of protecting groups, which are useful are for example FMOC, BOC, t-butyl esters, t-butyl ethers. The reactive group is used to build to attach specifically thereto (after the cleavage of the protecting group) another molecule.

The "localized area" is either known/defined by the construction of the micro-array or is defined during or after the detection and results in a specific pattern. A spot is the area where specific target molecules are fixed on their capture molecules and approved by a detector.

As used herein, the term "carrier" or "support" refers to any material that provides a solid or semi-solid structure and a surface allowing attachment of molecules. Such materials are preferably solid and include for example metal, glass, plastic, silicon, and ceramics as well as textured and porous materials. They may also include soft materials for example gels, rubbers, polymers, and other non-rigid materials. Preferred solid carriers are nylon membranes, epoxy-glass and borofluorate-glass. Solid carriers need not be flat and may include any type of shape including spherical shapes (e.g., beads or microspheres). Preferably solid carriers have a flat surface as for example in slides (such as object slides) and micro-titer plates, wherein a micro-titre plate is a dished container having at least two wells.

The expression "attached" describes a non-random chemical or physical interaction by which a connection between two molecules is obtained. The attachment may be obtained by means of a covalent bond. However, the attachments need not be covalent or permanent. Other kinds of attachment include for example the formation of metalorganic and ionic bonds, binding based on van der Waal's forces, or any kind of enzyme substrate interactions or the so called affinity binding. An attachment to the surface of a carrier or carrier may be also referred to as immobilization.

A "determinant" relates to a factor responsible for the development of resistance in P. aeruginosa, which may be acquired by the micro-organism via horizontal gene transfer and which actively counteracts the effect of an antibiotic. Particularly, the genes conveying

resistance to antibiotics, such as mexR, mexT, nfxB, mucA, parC, gyrA, exoU, exoS, exoT, pse, oxa, imp, vim, aac, aph and aad, which may be normally present on plasmid(s) or also may be incorporated in the genome of P. aeruginosa, are envisaged. Also virulent factors, such as e.g. genes involved in the synthesis of toxins and alginate are comprised by said term.

The terms "complementary" or "complementarity" are used in reference to polynucleotides (i.e., a sequence of nucleotides such as an oligonucleotide or a target nucleic acid) in the light of the base-pairing rules. Complementarity may be partial, in which only some bases of the nucleic acids are matched according to the base pairing rules. Alternatively, there may be a complete complementarity between the nucleic acids in such a way that there are no mismatches. The degree of complementarity between nucleic acid strands has significant effects on the stringency and strength of the hybridization between two different nucleic acid strands. Complementarity as used herein is not limited to the predominant natural base pairs. Rather, the term also encompasses alternative, modified and non-natural bases, including but not limited to those that pair with modified or alternative patterns of hydrogen. With regard to complementarity, it is important for some applications to determine whether the hybridization represents a complete or partial complementarity. If it is desired for example to detect the presence or absence of a particular DNA (such as from a virus, bacterium, fungi or protozoan), the only important condition is that the hybridization method ensures hybridization when the relevant sequence is present. Other applications in contrast, may require that the hybridization method distinguish between partial and complete complementarity, for example in the detection of genetic polymorphisms.

The term "homology" and "homologous" refers to a degree of identity. There may be partial homology or complete homology. A partially homologous sequence is one that is less than 100% identical to another sequence.

"Hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acids) is influenced by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, and the melting temperature of the

formed hybrid. Hybridization involves the annealing of one nucleic acid to another complementary nucleic acid, i.e., a nucleic acid having a complementary nucleotide sequence.

"Stringency" refers to the conditions, which are involved in a correct hybridization event, for example temperature, ionic strength, pH and the presence of other compounds, under which nucleic acid hybridizations are conducted. Under conditions of high stringency, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences. Thus, conditions of weak or low stringency are often required when it is desired that nucleic acids that are not completely complementary to one another be hybridized or annealed together.

A "marker" or "label" refers to any atom or molecule that may be used to provide a detectable (preferably quantifiable) effect and that can be attached to a nucleic acid. Markers may include colored dyes; radioactive labels; binding moieties such as biotin; haptens such as digoxgenin; luminogenic, phosphorescent or fluorogenic moieties; and fluorescent dyes alone or in combination with moieties that can suppress or shift emission spectra by the energy transfer of fluorescence. Markers may provide signals, which are detectable for example by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism and enzymatic activity. A marker may be a charged moiety (positive or negative charge) or may also have a neutral charge. They may include or consist of nucleic acid or protein sequence. Preferred markers are fluorescent dyes.

A "target" or "probe molecule" refers to a nucleic acid molecule to be detected. Target nucleic acids may contain a sequence that has at least a partial complementarity with at least a probe oligonucleotide.

"Probes" or "probe molecules" refer to nucleic acids, which interact with/ hybridize to a target nucleic acid to form a detection complex.

The term "signal probe" or "probe" relates to a probe molecule, which contains a detectable

moiety, which are already outlined above.

The term "nucleic acid" is meant to comprise any sequence of deoxyribonucleotides, ribonucleotides, peptido-nucleotides, including natural and/or artificial nucleotides.

The expression "sample" is meant to include any specimen or culture of biological and environmental samples or nucleic acid isolated therefrom. Biological samples may be animal, including human, fluid, such as blood or urine, solid or tissue, alternatively food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products. Environmental samples include environmental material such as surface matter, soil, water, industrial samples and waste, for example samples obtained from sewage plant, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. The sample may be used as such in the assay or may be subjected to a preliminary selection step, such as e.g. culturing the sample under conditions favoring or selecting for P. aeruginosa in said sample. Also, the nucleic acids contained in the sample may be isolated prior to performing the assay. In the presence of a multi-resistant P. aeruginosa in the sample the resulting nucleic acid sample will contain the target nucleic acid which may be isolated from the biological sample in any way known to the skilled person, including conventional isolation comprising lysis of the cellular material of the biological sample and isolation of DNA or RNA therefrom. In case the target nucleic acid is present in a low amount, the said nucleic acid may be subjected to PCR, preferably to a multiplex PCR, to specifically amplify the target nucleic acid prior to performing the assay.

A "nucleic acid sample" may be a polynucleotide or oligonucleotide of a variable length and is represented by a molecule comprising at least 5 or more deoxyribonucleotides, preferably about 10 to 1000 nucleotides, more preferably about 20 to 800 nucleotides and more preferably about 20 to 100 or even more preferred about 20 to 60. The exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage,

transport, or delivery of reaction reagents (e.g., oligonucleotides, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another.

As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., oligonucleotides, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another.

Detailed description of the present invention

According to an embodiment, the present micro-array comprises a carrier or support on which in the form of a specific pattern nucleic acids are immobilized. Said nucleic acids comprise sequences specific for at least 8 determinants of P. aeruginosa. For a correct determination of the presence of multi-resistant P. aeruginosa in a sample a number of at least eight determinants have proven to yield a doubtless, non-ambiguous result. Since due to single nucleotide polymorphisms (SNPs) contained in a particular determinant, said determinant has to be characterized by more than one nucleic acid sequence, so that more than one capture probe is required for particular determinants to provide a detectable hybridisation event under stringent conditions. In consequence, also number of nucleic acid capture probes corresponding to known SNPs is attached to the surface of the carrier of the present micro-array to act as the capture molecule for the particular determinant, thereby allowing the individual and unambiguous detection of each SNP of said determinant. The different capture probes (for the different SNPs) for one particular determinant may be attached to the carrier (e.g. spotted) on one localized area or on different ones.

Said immobilized nucleic acids comprise sequences specific for at least 8 determinants of P. aeruginosa, which sequences are preferably randomly selected from the group consisting of mexR, mexT, nfxB, mucA, parC, gyrA, exoU, exoS, exoT, pse, oxa, imp, vim, aac, aph and aad. Each of these determinants is detected either by a single capture probe or a set of two or

more capture probes, which number of capture probes depend from the number of SNPs said determinant embraces. For a correct and unambiguous identification of the strain and the detection of a multiresistant P. aeruginosa strain 8 determinants, which may include resistance genes and other genes conferring virulence to said strain, have proven to be sufficient without any requirements concerning the selection of the determinants. The detection of 9 or more determinants is preferred, since in this case more precise information about antibiotic determinants and other virulence factors are achieved. Thus, the present micro-array may also comprise nucleic acids probes specific for at least 9 to 11 determinants, more preferably at least 12 to 14 determinants, still more preferably at least 15 determinants and most preferably nucleic acids probes specific for 16 determinants.

The inclusion of P. aeruginosa specific control capture probes (oprI and gyrB) as well as capture probes for the detection of a broad range of gram-negative organisms (srv3) allows a more correct species identification.

The carrier or support of the present DNA micro-array may consist of different materials, preferably of glass, silicon, silica, metal, plastics or mixtures thereof prepared in format selected from the group of slides, discs, gel layers and/or beads. The carrier may also be a microplate or a slide and may consist of epoxy glass. A preferred support is for example an epoxy modified glass slide purchased by Elipsa AG, Berlin, Germany.

Preferably, the present micro-array has at least 100 molecules attached per square centimeter of the solid carrier. This density may be, however, higher and be adapted to the respective application of the micro-array, in that also other suitable applications may be performed, e.g. for the determination of resistances in other organisms different from P. aeruginosa and/or for the detection of resistance gene(s), which are unknown yet to play a role in P. aeruginosa. For example, the density of the nucleic acids probes attached per square centimeter of solid carrier amounts more preferably at least to 1.000, still more preferably at least to 5.000 and most preferably at least to 10.000 nucleotides per square centimeter.

Said specific pattern allows the mapping of each nucleic acid probe to a specific position on

said carrier and a specific analysis, in that the analysis of the results of the present micro-array is facilitated and non-ambiguous concerning the attribution of a particular spot to a previous attached nucleic acid probe.

Spacer molecules of any length may be arranged between the carrier and the nucleic acids applied on the carrier. The spacer may be for example polymer-based spacers, but may also consist of an alkane chain, or any derivatives thereof, of a suitable length, which comprises at each end respective functional groups for attachment to the solid support and the nucleic acid probe. Preferably, 15-thymidine spacers have been attached with one end to the surface of the support and with the other end to the 3'-terminal end of the respective nucleic acid to be immobilized.

According to another preferred embodiment, the present invention provides a method for the detection of multi-resistant P. aeruginosa strains in a sample material, using a micro-array for the detection of determinants like resistance genes and other genes conferring virulence.

The method comprises the step to obtain a sample material of interest. Prior to performing the method of the present invention the sample may be pre-treated e.g. centrifuging or filtering to separate non-soluble matter or selecting for P. aeruginosa in the sample. This may be achieved by e.g. culturing the sample under conditions favouring the growth of P. aeruginosa. Also, to improve performance, nucleic acids contained in the sample material may be isolated and/or amplified. The sample and/or the isolated/purified nucleic acid material is applied to the surface of the present micro-array. Said sample is now allowed to hybridize to the immobilized nucleic acids, the capture probes, for targeting at least 8 determinants of P. aeruginosa. By choosing suitable hybridisation conditions known to the skilled person, such as e.g. applying a certain stringency during hybridization and washing (cf. Maniatis et al., Molecular Cloning - A Laboratory Manual, First Edition, Cold Spring Harbor, 1982), only those nucleic acids will hybridize to the immobilized nucleic acids and/or remain bound during washing steps, which exhibit a high homology to the immobilized nucleic acids. The method further comprises detecting any hybridisation event, which will be indicative of the presence of a multi-resistant P. aeruginosa.

Said nucleic acids probes specific for targeting at least 8 determinants of P. aeruginosa are preferably randomly selected from the group consisting of mexR, mexT, nfxB, mucA, parC, gyrA, exoU, exoS, exoT, pse, oxa, imp, vim, aac, aph and aad. Each of these determinants is detected by a specific set of capture probes, which may comprise more than one nucleic acid probe in accordance to the number of SNPs said determinant embraces. For a correct and non-ambiguous indentification of the strain and the determination of a multiresistant P. aeruginosa strain 8 determinants, which include resistance genes and other genes conferring virulence to said strain, have proven to be sufficient without any requirements concerning the selection of the determinants. Preferably, the micro-array may also comprise nucleic acids specific for at least 9 to 11 determinants, more preferably at least 12 to 14 determinants, still more preferably at least 15 determinants and most preferably 16 determinants.

P. aeruginosa specific control probes (oprI and gyrB) may be included. Other controls are probes, which are capable to detect a broad range of gram-negative organisms (srv3) for a correct species identification.

The nucleic acid sample to be used for hybridizing to the immobilized nucleic acids consists preferably of oligonucleotides and/or polynucleotides of a length between 10 and 1000 nucleotides each, preferably shorter oligonucleotides/polynucleotides exhibiting a length of about 10 to 100 or between 20 to 60. The length may be obtained for example by the digestion of plasmid or genomic DNA with DNAse or preferably restrictions enzymes and facilitates the hybridisation.

The nucleic acid sample, which comprises oligonucleotides and/or polynucleotides, is preferably isolated from body tissues or fluids, particularly blood, suspected to contain P. aeruginosa, followed by the isolation and optional the amplification of the DNA and/or RNA contained therein by PCR techniques, such as a multiplex PCR, which allows the amplification of several DNA fragments in one PCR reaction. Such techniques are well known to the skilled person and may be also performed with commercial available kits.

The capture and the target nucleic acids may be present in a labeled form. The target nucleic acids may be labeled prior to performing the assay, by including a marker molecule into the molecule, e.g. during its amplification or isolation. Said marker molecule is preferably a fluorescent marker. Also the capture molecules may be labeled, in case of a fluorescent dye preferably with a dye exhibiting a different excitation and/or emittance wavelength, which allows a normalization of the experiment.

Methods for the detection of binding include e.g. surface plasmon resonance or detection of fluorescence at a localized area indicative of binding of a labelled molecule. Fluorescence may be detected e.g. via confocal laser induced fluorescence.

In another embodiment of the invention, a diagnostic kit is provided for the detection of P. aeruginosa infections.

Said kits either provides the nucleic acids specific for 16 determinants of P. aeruginosa, which determinants are selected from the group consisting of mexR, mexT, nfxB, mucA, parC, gyrA, exoU, exoS, exoT, pse, oxa, imp, vim, aac, aph and aad. Alternatively, the kit may also provide a micro-array as detailed above.

Additionally, the kit may also include the appropriate controls, in that probes are included specific for the gyrB, oprI and srv3 genes.

A typical automated processing of a micro-array according to a preferred embodiment of the present invention includes the use of three components. First, the micro-array or support respectively, second a reader unit and third means for the evaluation of the results, e.g. a suitable computer software. The reader unit comprises in general a movable tray, focussing lens(es), mirrors and a suitable detector, e.g. a CCD camera. The moveable tray carries the micro-array and may be moved to place the micro-array within the light path of one or more suitable light sources, e.g. a laser with an appropriate wavelength to excite a fluorescent compound. The evaluation program or software may serve for example to recognize specific patterns on the array or to analyse different expression profiles of genes. In this case, the

software searches colored points on the array and compares the intensity of different color spectra of the same point. The result may be interpreted by an analyzing unit and afterwards stored in a suitable file format for further processing.

As detailed above, the probe- and/or target- nucleic acids may be labelled each with a fluorescent dye and the intensity of the fluorescence at different wavelengths of each point is compared to the background. The detector, e.g. a photomultiplier or CCD array, transforms low light intensities to an amplifiable electrical signal. Other methods use different enzymes, which are covalently bound to the nucleotide by means of a linker molecule. The enzymatic colorimetry uses for example alkaline phosphatase and horseradish peroxidase as marker. By contacting with a suitable molecule, a detectable dye may be achieved. Other chemo-luminescent or fluorescent marker comprise proteins capable to emit a chemoluminescent or fluorescent signal, if irradiated with light of a discrete, specific wavelength, e.g. 488 nm for the green fluorescent protein. Radioactive markers are applied in case of low detection limits are required, but are due to their harmful properties not wide spread. Fluorescence marking is performed with nucleotides linked to a fluorescent chromophore. Combinations of nucleotides and fluorescent chromophore comprise in general Cy3 (cyanine 3)/ Cy5 (cyanine 5) labelled dUTP as dye, since they may be easily incorporated, the electron migration for fluorescence may be exited by means of customary lasers and they also have distinct emission spectra.

The hybridisation of micro-arrays follows essentially the conventional conditions of southern or northern hybridisations, which are well known to the skilled person. The steps comprise a pre-hybridisation, the intrinsic hybridisation and a washing step after hybridisation occurred. The conditions have to be chosen in such a way that background signals are kept low, minimal cross-hybridisation (in general a reduced number of mismatches) occurs and with a sufficient signal strength, which has to be proportional for some applications to the concentration of the target molecule.

The hybridisation event may be detected generally by two different kinds of array-scanners. One method employs the principle of the confocal laser microscopy, which uses at least one

laser to scan the array in point-to-point manner. Fluorescence is than detected by photomultipliers, which amplify the emitted light. The cheaper GGD basing readers use typically filtered white light for the excitation. The surface of the array is scanned with this method in sections, which allows the faster achievement of results of a lower significance.

Also the so-called gridding for the analysis of the results, in which an idealised model of the layout of the micro-array is compared with the scanned data to facilitate the spot definition. Pixels are classified (segmented) as spot (foreground) or background to produce the spotting mask. Segmentation techniques may be divided in fixed segmentation circle, adaptive circle segmentation, adaptive shape segmentation and histogram segmentation. The use of these techniques depends from the shape of the spots (regular, irregular) and the quality of the proximal arrangement of the spots.

Another issue is the intensity of the distinct spots, since the concentration of hybridised nucleotides in one spot is proportional to the total fluorescence of this spot. In particular, the overall pixel intensity and the ratio of the different fluorescent chromophores used (in case of Cy3 and Cy5, green and red) are important for the calculation of the spot intensity. Beneath the spot intensity, also the background intensity has to be taken into account, since various effects may disturb the fluorescence of the spots, for example the fluorescence of the support and of the chemicals used for the hybridisation. This may be performed by the so-called normalisation, which includes the above-mentioned effects and others like fluctuations of the light source, the lower availability/incorporation of the distinct marker molecules (Cy5 worse than Cy3) and their differences in emission intensities. Of importance for the normalisation is further the reference against which shall be normalized. In general, this may be a specific set of genes or a group of control molecules present on the micro-array.

The results may be further processed by means of the available software tools and according to the knowledge of bioinformatics.

The present invention provides a method, a micro-array and kit for the detection of P.

aeruginosa infections, helpful in reducing the spread of multi-resistant isolates and improve the treatment options of severe and often life-threatening P. aeruginosa infections. The present inventors could verify surprisingly in the course of their studies also the presence of a vim gene in a clinical isolate, which represents the first alarming occurrence of said determinant in connection with a multi-resistant P. aeruginosa strain in Germany.

It is to be understood, that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skilled in the art upon reviewing the above description. By way of example, the invention has been described preliminary with reference to the use of nucleic acids comprising sequences specific for the resistance and virulence determinants of P. aeruginosa. It should be clear that also other resistance and virulence determinants may be selected in dependence from the genetic development of multiresistant P. aeruginosa strains. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

Examples

A. Bacterial strains and culture conditions

The wild-type reference strain P. aeruginosa PAO1 was obtained from the ATCC (AT47085). The other P. aeruginosa strains were collected from patients at the Robert Bosch Hospital in Stuttgart, Germany. They were recovered from respiratory samples (n=51), swabs (n=5), urine (n=2) and faeces (n=2). All isolates were identified with the API 20NE system (bioMerieux, Marcy l'Etoile, France) and the NEG Breakpoint Combo Type 30 panel on the MicroScan WalkAway ®-96 SI system (Dade Behring, Liederbach, Germany). All bacterial strains were either routinely cultured at 37°C on Mueller-Hinton (MH) agar or grown in Luria Bertani broth (LB).

B. Antibiotic susceptibility testing

The antibiotic susceptibility was determined with the NEG MIC Type 30 panel on the

MicroScan WalkAway ®-96 SI system. The MICs were interpreted according to the NCCLS guidelines. The strains were tested for aztreonam (AZT), ceftazidime (CAZ), cefepime (CPE), piperacillin (PI), piperacillin/tazobactam (P/T), imipenem (IMP), meropenem (MER), levofloxacin (LVX), ciprofloxacin (CP), colistin (COL), gentamicin (GM), tobramycin (TO) and amikacin (AK).

C. DNA methods, PCR, labeling and sequencing

Chromosomal DNA was extracted with the QIAmp DNA Mini Kit, plasmid DNA with the QIAprep Spin Miniprep Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). A set of 4 multiplex PCRs (Tab. 1) was set up to amplify the sequences of interest from the chosen genes. The PCRs were carried out with the Advantage®-GC Genomic PCR Kit (BD Bioscience, San Jose, USA) according to manufacturer's instructions, for fluorescence labeling 5 µl of each 1 mM dNTP was used, for dCTP a 2:3 mixture of Cy3-dCTP and dCTP. The cycle reactions consisted of 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and sizes of PCR products were checked on a lab-on-a-chip Bioanalyzer 2100 electrophoresis with the DNA 1500 LabChip kit (Agilent, Böblingen, Germany). Sequencing was performed on the ABI PRISM® 310 Genetic Analyzer using the BigDye® Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, USA). The manufacturer's protocol was followed using the same primers as for the multiplex PCRs. Sequences were assembled, aligned and analyzed with the Lasergene software, Version 5.08 (DNAStar, Madison, USA).

D. Probe design

Oligonucleotide probes specific for positions affected by SNPs were designed to have a nucleotide responsible for perfect match/mismatch at a central position. A set of 4 oligonucleotide probes was designed for each particular SNP having identical sequence except that for the central base, which was one of the 4 possible nucleotides A, T, G or C. A reliable detection of mutations due to insertion or deletion of particular bases was achieved with two probes optimized either for wild-type sequence or mutated sequence. The presence of relevant genes was confirmed with a set of two probes for each gene. Both probes were

designed to represent the same sequence, one for sense direction and the other one for anti-sense direction. In order to keep all the probes within a certain thermal range for a simultaneously hybridization, the length of the capture oligonucleotides were varied between 17 and 24 bases.

E. Array fabrication

The oligonucleotide array consisted of 202 amino-modified capture probes containing a poly-(T) 15 spacer at the 5`-end. They were synthesized by MWG Biotech (Ebersberg, Germany) and resuspended in spotting buffer S1 (160 mM Na 2 SO 4 , 130 mM Na 2 HPO 4) to a final concentration of 20µM. The array layout is shown in Fig. 1. Each capture probe was spotted in triplicates on CreativeChip™ Oligonucleotide slides (Elipsa AG, Berlin, Germany) with the Microgrid II arraying system using MicroSpot 2500 pins (Biorobotics, Cambridge, UK). Spotted capture probes were covalently immobilized to the glass surface by incubation at 60 °C for 30 min in a drying compartment (Memmert, Schwabach, Germany). Blocking and cleaning of the fabricated slides until further use was performed according to the manufacturer's instructions.

F. Controls.

Several controls were included on the array:

a spotting control (5'-cyanine 5 [Cy3]-TTTTTTTTTTTTTTCTAGACAGCCACTCATA-3');

a positive hybridization control (5'-TTTTTTTTTTTTTGATTGGACGAGTCAGGAGC-3') complementary to a labeled oligonucleotide target (5'-Cy3-GCTCCTGACTCGTCCAATC-3'), which was spiked during hybridization; and

a negative hybridization control (5'-TTTTTTTTTTTTTTCTAGACAGCCACTCATA-3'). All these control sequences are unrelated to sequences found in bacterial species.

G. Fragmentation

In order to increase hybridization efficiency, the amplified and labeled target DNA was diluted to a concentration of 30 ng/µl in reaction buffer (40 mM Tris/HCl, pH 8.0, 10 mM $MgSO_4$, 1 mM CaCl2) and fragmented with DNAseI (11.5 mU/µl) (Invitrogen, Karlsruhe, Germany) at room temperature for 5 min. The reaction was stopped by addition of 3 mM

EGTA and incubation at 65 °C for 10 min.

H. Hybridization

400 ng fragmented target DNA with addition of control DNA (0.05 pmol) were hybridized under a 18 mm x 18 mm cover glas in 30 µl of 6x SSPE (1x is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [ph 7.7]), incubated in an Eppendorf Thermomixer Comfort (Eppendorf AG, Hamburg, Germany) at 55 °C for 1 h. After hybridization the slides were washed with 2x SSC, 0.1 % SDS then 2x SSC (1x is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.2x SSC, each time for 10 min at room temperature with agitation in a glass container. Finally, the slides were dried with air.

I. Data acquisition and processing.

The oligonucleotide arrays were scanned with a arrayWoRx Biochip Reader (Applied Precision, Marlborough, UK). The scanner settings for fluorescence signal acquisition were set to "High Precision" and 0.2 s acquisition time. The image processing and calculation of signal intensity was performed with the ArrayPro software (MediaCybernetics, San Diego, USA). The net signals were obtained by substraction of the local background from the absolute density signal. The global background area is defined as the area between the spots of the array. The software calculates a minimum, maximum and average background value from the global background data.

J. Susceptibiliy profile of the isolates

The test collective of *P. aeruginosa* isolates was recovered from three intensive care units (ICU) or other hospital wards as far as an unusual multidrug resistance was observed. Most isolates were obtained from respiratory samples, followed by wound swabs and urine (Tab. 3). An overview of the antibiotic susceptibilities of the isolates are shown in Tab. 4. Susceptibility profiles of the tested strains showed a typical distribution for an ICU. Ten *P. aeruginosa* isolates showed a concomitant antibiotic resistance against one or more antibiotics from the group of aminoglycosides, fluoroquinolones, cephalosporines and carbapenemes. Among the tested substances colistin was most effective substance with a

resistance rate of 0 % and imipenem the endmost effective with a susceptibility rate less than 55 %.

K. Array set-up

The two major features of array based test systems are sensitivity and specificity. In contrast to usually methods used to discriminate specific from unspecific signals based on an internal DNA standard we defined a cut-off value based on the background fluorescence intensity. The cut-off value for a specific, positive fluorescence signal was set to 1.5 times of the minimal background signal value. Each signal, regardless perfect match or mismatch was considered as positive, if the absolute fluorescence intensity exceeded this value. Everything below was considered as unspecific and was not considered in any subsequent analyzes. Fig. 2 shows a typical array experiment with hybridization patterns of the reference strain PAO1 (a) and *P. aeruginosa* isolate No. 23 (b). The hybridization of an array with target DNA obtained from PAO1 revealed a minimal background fluorescence intensity of $4.86 * 10^9$ RFU (relative fluorescence unit). The signal cut-off was set to $7.29 * 10^9$ RFUs. For *P. aeruginosa* isolate No. 23 the respecive values were $5.06 * 10^8$ RFU and $7.59 * 10^8$ RFU.

The second major feature of an array is specificity defined by the ability to discriminate between mimatch and perfect match signal. The highest fluorescence signal of each SNP or insertion/deletion position was considered as potential perfect match position (PM). The other signals of probes specific for particular mutation position were considered as potential mismatch positions (MM) and normalized to the PM value. The potential perfect match signal was set to the value of 1.0 and the mismatch signals were adjusted accordingly. The ratio of MM/PM ranged from 0.0 to 1.0. The relative intensity value of particular probe was considered as specific, if MM/PM ratio for that probe did not exceed the value of 0.7. The MM/PM ratio for all of tested capture probes remained under 0.7.

Every gene which is subject to mutations was sequenced for both strands in order to determine the correlation between array deduced genotype and sequencing based genotype. In all cases, the highest array signals for the different SNP, insertion or deletion positions corresponded to the perfect match position according to the underlying genotype determined

by sequence analysis. Also the presence of plasmid or integron encoded resistance genes not subject to mutations could be verified via PCR and sequencing analysis.

L. Genotype analysis by array

The differential comparison of the two hybridization patterns showed a discrepant perfect match signal for positions 327, 377 and 384 in the *mexR* gene, position 248 in the *gyrA* and 240 in the *parC* gene (Fig. 3). All differences in perfect match signal were a matter of single nucleotide polymorphism. The mutations in *mexR* at position G327A and G384A were silent and did not cause an amino acid exchange, but mutation T377A causing a Val126Glu exchange was responsible for respective antibiotic resistance. The mutation C248T (Ser83Leu) in the gyrase gene *gyrA* as well as mutation C240T (Ser80Leu) found in the topoisomerase IV gene *parC* leads to respective amino acid substitution that is responsible for fluoroquinolone resistance. In addition, specific fluorescence signals from isolate No. 23 indicated the presence of a *vim, aac(6')-Ib* and *aadA1* gene. There were no additional differences as analyzed by the presented array in genotypical characteristics of clinical isolate No. 23 and reference *P. aeruginosa* strain PAO1.For *oprI, srv3, exoS, exoT, aadA1* and *aac(6')-Ib* the signals for sense and anti-sense probes are displayed, showing that usually one get signals above the cut-off for both probes.

The 60 *P. aeruginosa* isolates were tested with the array applying the same hybridization conditions. Tab. 4 and Tab. 5 show a summary of the array analysis, covering the genes which are affected by mutations or are aquired by plasmid acquisition. The presence of such genes may contribute to antibiotic resistance or virulence.

The distribution of the mutations indicate the existance of hot spots at sequence positions 327, 377 and 384 for the *mexR* gene, 305 for the *nfxB* gene, 197 and 212 for mexT and 248 for the *gyrA* gene, respectively. 22 isolates harbored 3 mutations in *mexR* at position 327, 377, 384. Furthermore, the array analysis of 60 *P. aeruginosa* clinical isolates revealed as well the presence of plasmids or integrons encoding for antibiotic resistance genes (*imp, vim, oxa, aad, aac*). Concerning the group of aminoglycoside modifying enzymes, the following genes were detected: *aac(6')-Ib, aac(3)-Ia,-Ib and –II, aadA1, -2, aadB* and *aph(3')*. In the group of β-lactamases, 8 *imp* and one *vim-1* gene could be determined. However, the most

frequent β-lactamase genes found in 17 isolates of the collective belong to the *oxa*-family, 13 of them the ESBL *oxa-14* gene. The array data revealed the presence of virulence factors, *exoS* and *exoT* genes were found in 47 isolates, 13 isolates harbored an *exoU* gene. This is consistant with the usual frequencies reported for these genes. E*xoS* and *exoT* were reported to occur with a frequency of about 68 %, *exoU* with 28 %, respectively (78 % and 22 % respectively in our collective).

M. Interarray variability

In order to determine if the ratio of perfect match to mismatch signal intensity after hybridization with target DNA from a set of different amplification/labeling reactions is reproducable, the array was hybridized four times with DNA obtained from PAO1. However, due to different dye incorporation and fragmentation efficiency the net intensities varied for some positions for a factor higher than three. Therefore, the relative intensity was calculated. After normalization, the overall interarray variability was 8.7 %. In any case, the relation between the 4 probes specific for a single SNP position was not changed from an array to another.

O. Array signal-genotype correlation

Every gene, which is subjected to mutations was sequenced for both strands in order to determine the correlation between array deduced genotype and sequencing based genotype. In all cases, the highest array signals for the different SNP, insertion or deletion positions corresponded to the perfect match position according to the underlying genotype determined by sequence analysis. Also the presence of plasmid or integron encoded resistance genes not subject to mutations could be verified via PCR and sequencing analysis.

P. Correlation of array based genotype and phenotype

The Fig. 4 shows a detail of the array analyses of three P. aeruginosa isolates that were collected from the same patient within a time period of three weeks. The first isolate was phenotypically susceptible to ciprofloxacin and tobramycin. The subsequent isolate, collected one week after the first isolate, was phenotypically already resistant against ciprofloxacin but

still susceptible for tobramycin. Finally, the last of the three isolates then showed phenotypical resistance for ciprofloxacin and tobramycin. A retrospective array analysis of these three isolates correlated well with the resistance phenotype. However, due to higher sensitivity it was possible to detect resistance relevant genes and mutations even in the first isolate (Fig. 4). All isolates carried a mutation at position 248 in gyrA (levofloxacin and ciprofloxacin resistance) and harbored an aac(6')-Ib (tobramycin and amikacin resistance) and aadA1 gene (gentamicin and tobramycin resistance).

In order to compare genotype and phenotype based resistance profiles, we calculated a cummulative resistance rate against different groups of antibiotics based on the array data (Tab. 6). Since one isolate may harbour one or more mutations or resistance genes which may confer resistance to a certain antibiotic at the same time, the cummulative resistance rates predicted from our array data were 5 to 10 % higher as compared with the phenotypic determined susceptibility rates.

Tab. 1 primers used for amplication

Name	Covered genes	Primer	Sequence (5 ' -3 ')	Product Size	GenBank accession number	Multiplex PCR
MexR	mexR	MexR for MexR rev	GATGCCCGCGCTGATGG AGGCACTGGTCGAGGAGATG	390	AE004479	I
MexT	mexT	MexT for MexT rev	ATGCCTGTCAGTGATCCTATGC CGGGTCTCGAACGGTGGGTCCTC	935	AE004676	
NfxB	nfxB	NfxB for NfxB rev	GCGACGCTGAAGGAACTGG CCGGGCGGTACTGGAATA	240	AE004874	
MucA	mucA	MucA for MucA rev	CAGCTTGCGGCGAGGATGC GTACCACTGACGGCGGATTGTTGC	454	AE004511	
parC	parC	parC for parC rev	CTGGATGCCGATTCCAAGCAC GAAGGACTTGGGATCGTCCGG	186	AB003428	
gyrA	gyrA	gyrA for gyrA rev	GACGGCCTGAAGCCGGTGCAC GCCCACGGCGATACCGCTGGA	417	AE004741	
ExoU	exoU	ExoU for ExoU rev	CCGTTGTGGTGCCGTTGAAG CCAGATGTTCACCGACTCGC	135	U97065	II
ExoS	exoS	ExoSfor ExoS rev	GCGAGGTCAGCAGAGTATCG TTCGGCGTCACTGTGGATGC	119	AE004801	
ExoT	exoT	ExoT for ExoT rev	AATCGCCGTCCAACTGCATGCG TGTTCGCCGAGGTACTGCTC	154	AE004444	
GyrB	gyrB	GyrB for GyrB rev	CCTGACCATCCGTCGCCACAAC CGCAGCAGGATGAAGACGCC	329	AB005881	
OprI	oprI	OprI for OprI rev	GCTCTGGCTCTGGCTGCT AGGGCACGCTCGTTAGCC	197	AE004712	
Srv3	16S rRNA	Srv3 for Srv3 rev	CGGNCCAGACTCCTACGGG TTACCGCGGCTGCTGGCA	204	AE004949 II	
Pse	carb-1,2,3,4	Pse for Pse rev	GCTAAATTACTATGATGCTGAG TATTGCCTTAGGAGTTGTCG	327	S46063, U14749	III
Oxa	Oxa-5,7,10,11,13,14,16,17,19,28	OxaI for OxaI rev	CAGAGAAGTTGGCGAAGTAAGAAT AACCCACCCAACCCACCAT	307	AF347074, U37105, Z22590, U59183 L38523, AF043100, AF060206, AF043381, AF231133	
	oxa-2,3,15,20	OxaII for OxaII rev	GCTCGGCGCTATTTGAAGAA GCGCAGCGTCCGAGTTGA	415	AJ295229, L07945, U63835, AF024602	
Imp	Imp-1,7,9, 10, 11	Imp for Imp rev	GACACTCCATTTACTGCTA ATTCAGATGCATACGTGGGGATAG	160	X98393, AY625689, AF318077, AY033653, AB074434, AB074437,AB074433	
Vim	Vim-1,2	Vim for Vim rev	TGATACAGCGTGGGGTGCGAAAAA GTGCCCCGGAATGACGAACTGTG	472	AJ291609, AF263519, AJ295229	
Aac	Aac(6')-Ib	Aac(6')-Ib for Aac(6')-Ib rev	CTCGAATGCCTGGCGTGTTTGA GTGGTGGGGCGGAGAAGAAGC	439	X60321, AF043381, U59183, AF231133, AF315351, AF315786	IV
	Aac(6')-II	Aac(6')-II for Aac(6')-II rev	ACTGGTCTATTCCTCGCACTCCTG CCCCCATAACTCTTCGCCTCAT	288	AF162771, M29695, AF318077	
	Aac(3)-Ia	Aac(3)-Ia for Aac(3)-Ia rev	GCCGGAGACTGCGAGAT GCAGTCGCCCTAAAACAAA	241	U12338	
	Aac(3)-Ib	aac(3)-Ib for aac(3)-Ib rev	ACGCTTCAGGTGGCTAATC ACAAAGTTAGGTGGCTCAATG	345	L06157	

	Aac(3)-II	aac(3)-II for aac(3)-II rev	TTCCCCCAAGGCGTGACC GCATACGCGGAAGGCAATAAC	424	AF466526	
Aph	Aph(3')-IIb	Aph(3')-IIb for Aph(3')-IIb rev	GAAGAACTCGTCCAATAGCCTGAA GCGACGCCTGCCTGCCAAATC	224	X90856	
Aad	aadA1	AadA1 for AadA1 rev	TATCAGAGGTAGTTGGCGTCAT TTCAGGAACCGGATCAAAGAGT	440	AJ291609, AJ295229	
	aadA2	AadA2 for Aada2 rev	TCAGGAACCGGGTCAAAGAAT GAGCGCCATCTGGAATCAAC	416	U12338	
	aadB	AadB for AadB rev	CGGCACGCAAGACCTCAA GCTTGGTGGGCAGACGAA	241	AF078527, AF133699 IV	

Tab. 2 Origin and source of the test collective of 60 clinical P. aeruginosa isolates

	ICU-Internal Medicine	ICU-General Surgery	ICU-Cardiac Surgery	Other wards	All
Bronchoalveolar lavage				1	1
Tracheal sectretion	5	9	18	1	33
Swab Pharynx/Nose	2	2	4	2	10
Sputum	3			4	7
Swab Wound	1	3		1	5
Faeces	1			1	2
Urine				2	2
Total	12	14	22	12	60

Tab. 3 Antimicrobial susceptibilities of P. aeruginosa test collective

Antibiotic	Number of resistant isolates out of 60	% of resistant isolates out of 60
Levofloxacin	26	43
Ciprofloxacin	24	40
Gentamicin	22	37
Tobramycin	6	10
Amikacin	10	17
Piperacillin	21	35
Tazobac	20	33
Ceftazidim	20	33
Cefepim	19	32
Aztreonam	22	36
Imipenem	27	45
Colistin	0	0

Tab. 4 Mutations found by array analysis in 60 P. aeruginosa isolates

Gene	mexR					nfxB			mexT		gyrA	parC	mucA	
Mutation locus	80	208	327	377	384	115	303	305	197	212	248	240	362	440
Number of isolates out of 60	2	6	24	24	22	1	3	9	13	10	19	3	2	4

Tab 5. Antibiotic resistance mediating genes found by array analysis in 60 P. aeruginosa isolates

Gene	Imp	Vim	Oxa	aadA		aadB	Aac(3)			Aac(6 ')		exoS	exoT	exoU
Subtype				1	2		Ia	Ib	II	Ib	II			
Number of isolates	8	1	17	5	1	4	4	2	7	6	0	47	47	13

Tab. 6 Comparison of phenotypic resistance rate and cumulative resistance prediction based on genotype determined by array analysis

Antibiotic	Number of resistant isolates out of 60	% of resistant isolates out of 60	60 MIC [µg/ml]	Cumulative resistance predicttion deduced from array
Levofloxacin Ciprofloxacin	26 24	43 40	>4 >2	52%
Gentamicin Tobramycin Amikacin	22 6 10	37 10 17	>8 >8 >32	45% 17% 20%
Piperacillin Tazobac	21 20	35 33	>64 >64	38%
Ceftazidim	20	33	>16	40%
Cefepim Aztreonam	19 22	32 36	>16 >16	40%
Imipenem	27	45	>8	50%
Colistin	0	0		0%

Tab. 7 Susceptibility profile for ciprofloxacin and tobramycin from 3 consecutive Pseudomonas aeruginosa isolates from the same patient

Isolate 1	Isolate 2	Isolate 3	Antibiotic
S	R	R	Ciprofloxacin
S	S	R	Tobramycin

Table 8 sequences of the capture probes and the positions of the respective SNPs

Gene	Sequence position (Name)*	Probe sequence** (5'-3')	Wild-type N***	Mutation N****	Description*****	Length[bases]
mexR	165 mexR se	tcccaggtcccNcaggttcag	C	G	Gln55His	21
	170 mexR se	tggcgtcccNggtcctgcag	A	C	Leu57Arg	20
	208 mexR se	ggatcttccNggtgatcagt	G	A	Arg70Gln	20
	264 mexR se	cgctggtccNtggggttg	C	G	Ser88Arg	18
	281 mexR se	aggaagagcNggaagctgcg	T	G	Gln94Pro	20
	320 mexR se	gcctccgcaNgctggtggat	T	G	His107Pro	20
	327 mexR se	atgatggcNtccgcatgctg	C	A	Glu109Asp	20
	377 mexR se	cttgttccNccgggggtgag	T	A	Val126Glu	19
	384 mexR se	cagggtggcNtgttcctcc	C	A	Gln128His	19
	65 mexR se	ctggatgcgccggtccgcac		CG ins	frameshift/stop	20
	65 mexR WT se	ctggatgcgccgtccgcac			wild-type	18
	69 mexR se	cgctctggatggcgcgtccg		C ins	frameshift/stop	20
	69 mexR WT se	cgctctggatgcgcgtccg			wild-type	19
	80 mexR se	caatcgagcatcgctctgga		T ins	frameshift/stop	20
	80 mexR WT se	caatcgagctcgctctgga			wild-type	19
	47-57 mexR se	gcgtccgcacccgccatcag		Δ10N	frameshift/stop	20
	47-57 mexR WT se	tgctggaagaccgccatcag			wild-type	20
	261-272 mexR se	ctggaagctgggttgcgct		Δ10N	frameshift	20
	261-272 mexR WT se	gtcgctgggattgcgctcg			wild-type	19
	294-300 mexR se	ggatggccagcccgtgagga		Δ10N	frameshift	20
	294-300 mexR WT se	cagcccctcgtcggtgag			wild-type	18
	367-377 mexR se	ggcctgttcccggggcaaac		Δ10N	frameshift/stop	20
	367-377 mexR WT se	ggggtgagcgggcaaaca			wild-type	18
	382-387 mexR se	gcaccagggtttccaccggg		Δ10N	frameshift	20
	382-387 mexR WT se	accagggtggcctgttccac			wild-type	20
mexT	197 mexT se	cgcgggtcaNgctgcgttcg	G	C	Leu65Val	20
	212 mexT se	acagtttctNtgcggcgcgg	C	T	Glu71Lys	20
	733 mexT se	aggtcgccggNgaaggacacca	C	A	Ala245Ser	22
	413 mexT se	atcaatagaagttggcgcg		ΔT	frameshift/stop	19
	413 mexT WT se	atcaatagatagttggcgcg			wild-type	20
nfxB	124 nfxB se	ccgcagaagcNgtgcagggt	G	T	Arg41Ser	20
	260 nfxB se	tgggtgaggNgttccttgat	T	G	His87Pro	20
	105 nfxB se	ggccttgctctacgccggccg		C ins	frameshift	21
	105 nfxB WT se	ggccttgcttacgccggccg			wild-type	20
	115 nfxB se	ggtgcaggggccttgct		ΔT	frameshift/stop	18
	115 nfxB WT se	ggtgcagggtggccttgct			wild-type	19
	188 nfxB se	tgatctggttccagtacgg		C ins	frameshift	19
	188 nfxB WT se	tgatctggttcagtacgg			wild-type	18
	303 nfxB se	tccgggcggactggaatacc		ΔT	frameshift	20
	303 nfxB WT se	tccgggcggtactggaatacc			wild-type	21
	305 nfxB se	tccgggcgggtactggaata		G ins	frameshift	20
	305 nfxB WT se	tccgggcggtactggaata			wild-type	19
gyrA	248 gyrA A	tagaccgcgNtgtcgccgtg	G	A	Ser83Ile	20
	260 gyrA A	cacgatggtgNcgtagaccgc	T	G	Asp87Tyr	21
parC	240 parC A	tagcaggccNagtcgccgtg	G	A	Ser80Leu	20
	251 parC A	ccatggcctNgtagcaggcc	C	T	Glu84Lys	20
exoS	exoS se	cttcaccaggccatccgc				18
	exoS as	gcggatggcctggtgaag				18
exoU	exoU se	gaaatcaccgcgctcgc				17
	exoU as	gcgagcgcggtgatttc				17
exoT	exoT se	aagtgctccaccaggcatc				20
	exoT as	gatggcctggtggagcactt				20
oprI	oprI se	cgtcttcggtagcggtcag				19
	oprI as	ctgaccgctaccgaagacg				19
gyrB	gyrB se	ctgaagtggatgttgctgaaggtc				24
	gyrB as	gaccttcagcaacatccacttcag				24
16S rDNA	srv3 se	ttactgcccttcctcccaactta				19
	srv3 as	taagttgggaggaagggcagtaa				19
mucA	359 mucA se	gtcccctgttNcgccatttgc	C	T	Thr120Asn	21
	362 mucA se	gtggtcccctNttgcgccatt	C	T	Thr121Asn	21
	377 mucA se	agggcgatcNgcgggggtggtc	C	T	Leu126Gln	21
	431 mucA se	gccccctgctNttcgctgtag	C	T	Ala144Glu	21
	434 mucA se	ggcgcccccctNctcttcgctg	C	T	Pro145Gln	21
	446 mucA se	gtgatcacctNcggcgccccc	C	T	Thr149Asn	21
	167 mucA se	agggtaggctccgcggtgcat		C ins	frameshift/stop	21
	167 mucA WT se	agggtaggctcgcggtgcat			wild-type	20
	371 mucA se	atctgcgggggtcccctgt		ΔT	frameshift/stop	20
	371 mucA WT se	atctgcgggtggtcccctgt			wild-type	21
	407 mucA se	ccagcacggcacgggccttc		A ins	frameshift/stop	21
	407 mucA WT se	ccagcacggccgggccttc			wild-type	20
	440 mucA se	acctgcggcgcccctgctct		ΔC	frameshift/stop	20
	440 mucA WT se	acctgcggcgcccctgctct			wild-type	21
	471 mucA se	cgggtatcgcatggacgagga		A ins	frameshift/stop	21
	471 mucA WT se	cgggtatcgctggacgagga			wild-type	20

Gene	Sequence position (Name)*	Probe sequence** (5'-3')	Wild-type N***	Mutation N****	Description*****	Length[bases]
aph(3')	aph(3')-IIb se	gaagaactcgtccaatagcctgaa				24
	aph(3')-IIb as	ttcaggctattggacgagttcttc				24
aadB	aadB se	atgtgctttgtaggccagtcca				22
	aadB as	tggactggcctacaaagcacat				22
aadA	aadA1 se	tttcatcaagccttacggtcacc				23
	aadA1 as	ggtgaccgtaaggcttgatgaaa				23
	aadA2 se	ggtgacttctatagcgcggagc				22
	aadA2 as	gctccgcgctatagaagtcacc				22
aac(6')	aac(6')-Ib se	catacccaatcggctctccat				21
	aac(6')-Ib as	atggagagccgattgggtatg				21
	aac(6')-II se	aacgatgtgcggccggt				17
	aac(6')-II as	accggccgcacatcgtt				17
aac(3)	aac(3)-II se	gttattgccttccgcgtatgc				21
	aac(3)-II as	gcatacgcggaaggcaataac				21
	aac(3)-Ib se	ctattgctgttccgcggtca				20
	aac(3)-Ib as	tgaccgcggaacagcaatag				20
	aac(3)-Ia se	aactcacgaccgaaaagatcaaga				24
	aac(3)-Ia as	tcttgatcttttcggtcgtgagtt				24
pse	417 pse se	atcacttgNgtcatagttg	T	A C	*pse-1, 2, 3* *pse-4*	20
	555 pse se	caaatcaccNagcttaccttca	T	A C	*pse-4* *pse-1, 2, 3*	22
bla-imp	imp se	gagaattaagccactctattcc			*imp-1, 7, 9, 10, 11*	22
	imp as	ggaatagagtggcttaattctc			*imp-1, 7, 9, 10, 11*	22
bla-vim	vim se	atcaacgccgccgacgc			*vim-1, 2, 4*	17
	vim as	gcgtcggcggcgttgat			*vim-1, 2, 4*	17
oxa (group 1)	428 oxa1 se	gatattctggNtgccatagga	A	T C	*oxa-10, 13, 14, 16, 17, 19, 28* *oxa-11*	21
	521 oxa1 se	atttatttaaaNatagagactc	G	A T	*oxa-13, 19, 28* *oxa-10, 11, 14, 16, 17*	22
oxa (group 2)	449 oxa2 se	cttgtcgaaggaNcggcgttgc	A	T C	*oxa-2, 3* *oxa-15*	22
	oxa2 se	gctcctgcgccgagattgc			*oxa-2, 15*	19
	oxa2 as	gcaatctcggcgcaggagc			*oxa-2, 15*	19
	oxa2 3 se	ctgttcttgtgccgcgatagc			*oxa-3*	21
	oxa2 3 as	gctatcgcggcacaagaacag			*oxa-3*	21

*se: sense probe; as: anti-sense probe; WT: wild-type

** N: Variable nucleotide in a capture probe for a SNP position, can be eiter A, T, G or C. On the array all four possible nucleotides are each represented with a capture probe. The indicated nucleotides are based on the capture probe sequence.

*** N represents the respective nucleotide in the wild-type strain *P. aeruginosa* PAO1

**** N represents the respective nucleotide for a SNP position in the mutant sequence; Δ: deletion; ins: insertion

***** For SNPs, deletions or insertions either the respective amino acid change, frameshift, occurance of a premature stop codon or change of mutated gene compared to the parental gene is indicated.

Claims

1. A micro-array comprising a carrier and immobilized thereon in the form of a pattern nucleic acids comprising sequences specific for at least 8 determinants of Pseudomonas aeruginosa.

2. The micro-array according to claim 1, wherein said at least 8 determinants of Pseudomonas aeruginosa are selected from the group consisting of mexR, mexT, nfxB, mucA, parC, gyrA, exoU, exoS, exoT, pse, oxa, imp, vim, aac, aph and aad.

3. The micro-array according to claim 1, wherein the micro-array also includes controls.

4. The micro-array according to claim 3, wherein the controls are selected from the group consisting of gyrB, oprI, srv3.

5. The micro-array according to claim 1, wherein said carrier consists of glass, metal or plastics.

6. The micro-array according to claim 5, wherein said carrier consists or epoxy glass.

7. The micro-array according to claim 6, wherein said carrier is a microplate or a slide.

8. The micro-array according to claim 1, wherein the surface of said carrier comprises an area of at least 1 square centimetre.

9. The micro-array according to claim 1, wherein the nucleic acids are present on the carrier at a density of at least 100 molecules per square centimetre.

10. The micro-array according to claim 1, wherein said specific pattern allows the mapping of each nucleic acid to a specific position on said carrier and a specific analysis.

11. The micro-array according to claim 1, wherein said nucleic acids are immobilized via a spacer molecule.

12. A method for determining the presence of Pseudomonas aeruginosa in a sample comprising the steps of:

a) providing a micro-array comprising a carrier and immobilized thereon in the form of a pattern nucleic acids comprising sequences specific for at least 8 determinants of Pseudomonas aeruginosa,

b) contacting a sample to be analyzed with the micro-array under conditions allowing hybridization of complementary strands; and

c) determining, whether hybridisation occurrs.

13. The method according to claim 12, wherein said at least 8 determinants of Pseudomonas aeruginosa are randomly selected from the group consisting of mexR, mexT, nfxB, mucA, parC, gyrA, exoU, exoS, exoT, pse, oxa, imp, vim, aac, aph and aad.

14. The method according to claim 12, wherein the micro-array also includes controls.

15. The method according to claim 12, wherein the controls are selected from the group consisting of gyrB, oprI, srv3.

16. The method according to claim 12, wherein said sample contains nucleic acids comprising oligonucleotides and/or polynucleotides, having a length of about 10 to 100 nucleotides.

17. The method according to claim 16, wherein said oligonucleotides and/or polynucleotides are isolated from body tissues or fluids, particularly blood, suspected to contain Pseudomonas aeruginosa.

18. The method according to claim 16, wherein said nucleic acids are labelled with a marker molecule.

19. The method according to claim 18, wherein said marker molecule is selected from the group consisting of cyanine dyes, preferably Cy3 and/or Cy5, renaissance dyes, preferably ROX and/or R110, and fluorescent dyes, preferably FAM and/or FITC

20. A diagnostic kit for the detection of Pseudomonas aeruginosa infections, comprising nucleic acids specific for at least 8 determinants of Pseudomonas aeruginosa and/or a micro-array comprising a carrier and immobilized thereon in the form of a pattern nucleic acids comprising sequences specific for at least 8 determinants of Pseudomonas aeruginosa; and optionally buffers.

21. The kit according to claim 20, wherein the determinants are selected from the group consisting of mexR, mexT, nfxB, mucA, parC, gyrA, exoU, exoS, exoT, pse, oxa, imp, vim, aac, aph and aad.

22. The kit according to claim 20, wherein controls are included.

Fig. 1

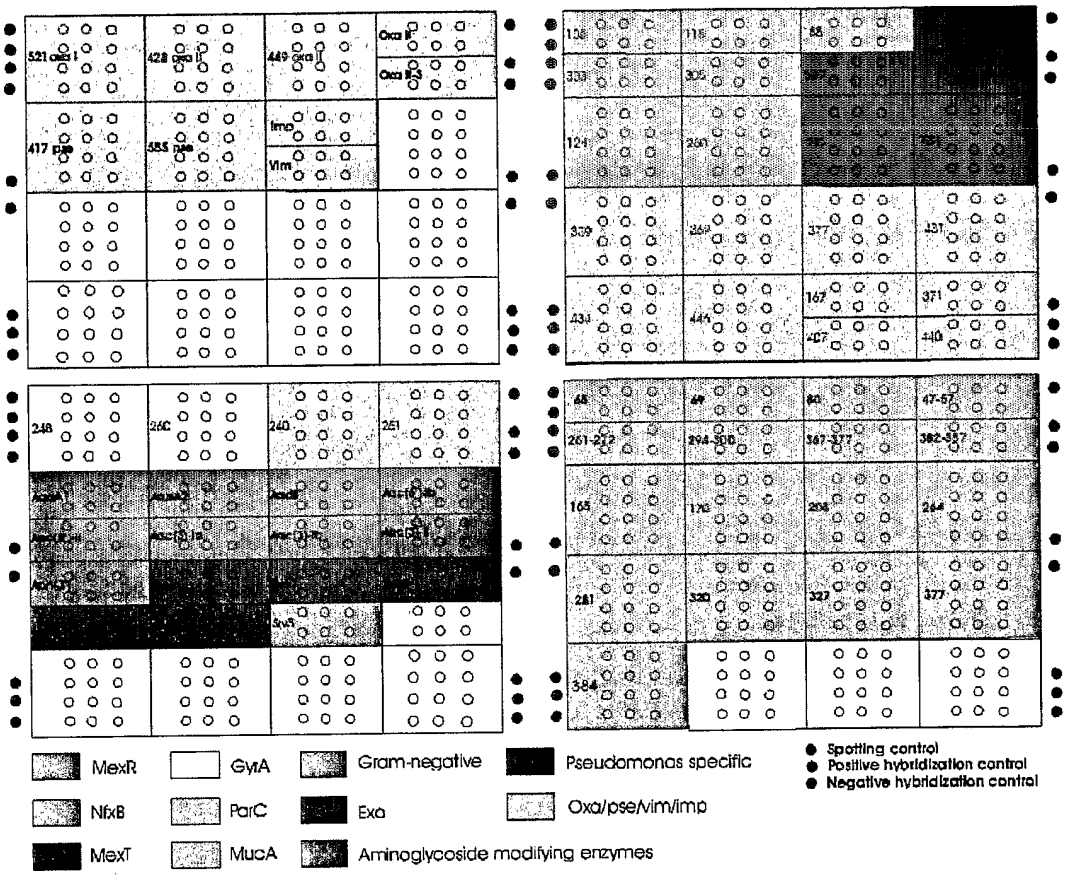
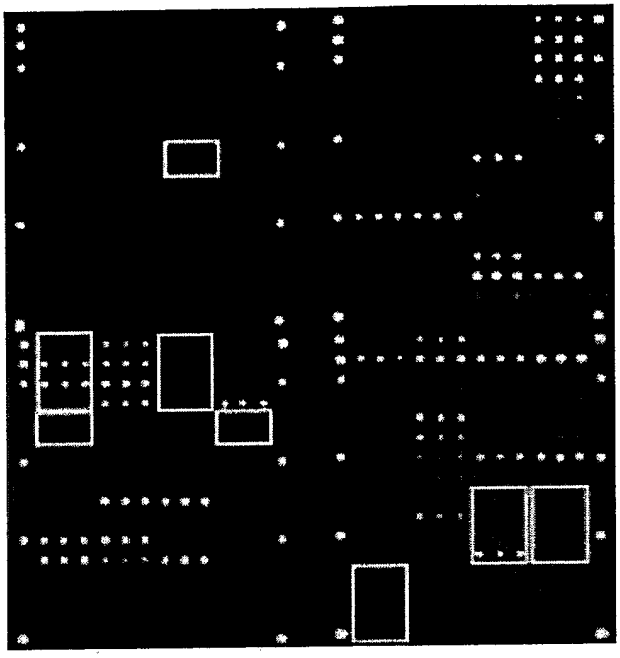
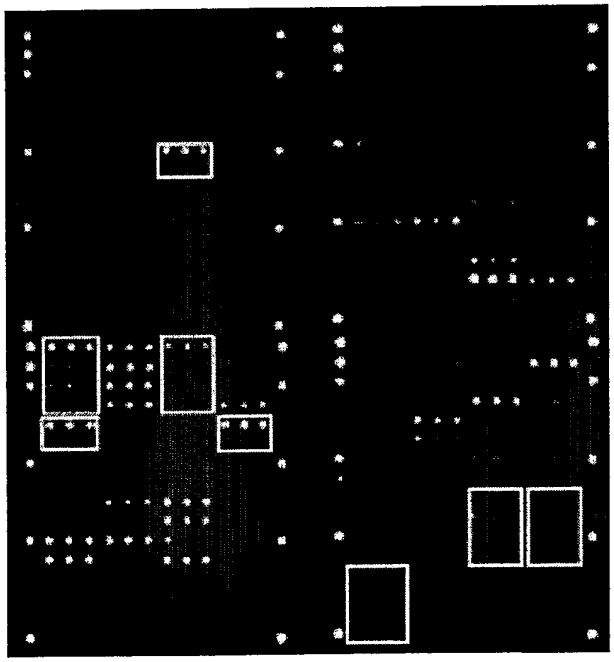

MexR
GyrA
Gram-negative
Pseudomonas specific
NfxB
ParC
Exo
Oxa/pse/vim/imp
MexT
MucA
Aminoglycoside modifying enzymes
Spotting control
Positive hybridization control
Negative hybridization control

Fig. 2



a



b

Fig. 3

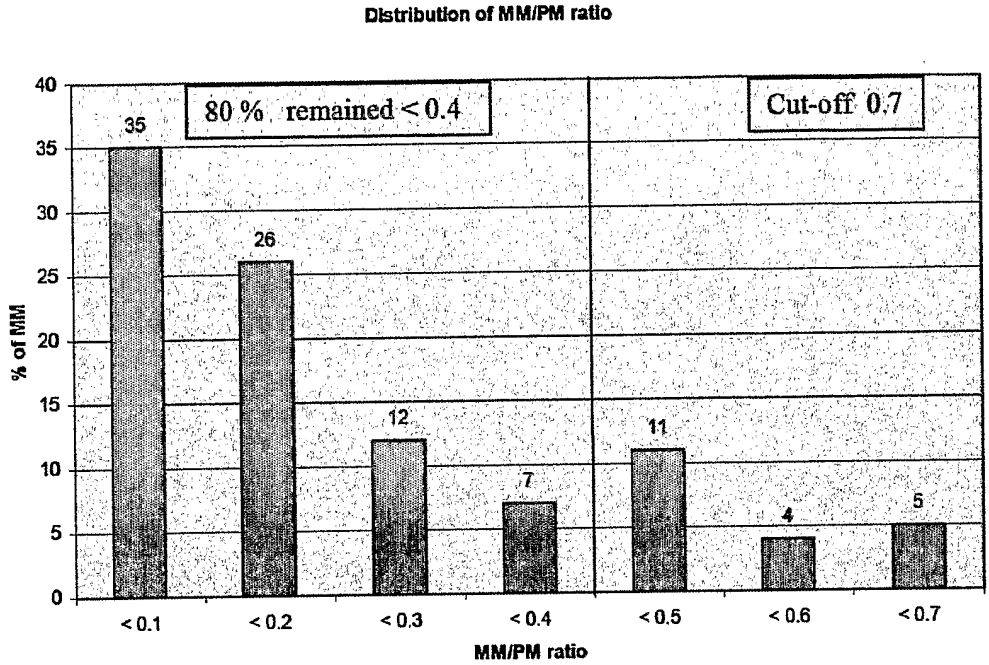

Distribution of MM/PM ratio
80 % remained < 0.4
Cut-off 0.7
% of MM
MM/PM ratio
35
26
12
7
11
4
5
< 0.1
< 0.2
< 0.3
< 0.4
< 0.5
< 0.6
< 0.7

Fig. 4