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ABSTRACT BOOKLET

Non-cognate Hybridization Systems (NCHS): an Open Strategy to Identify the Genetic Background of the Analyzed Strains

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Efficient and rapid microarray-based detection methods are required for assessing the presence of known pathogens, but also for identifying and characterizing new/unexpected or genetically engineered ones. We have developed a “non-cognate hybridization system” (NCHS) that consists in a novel microarray approach coupled to specific analytical tools. This approach should provide enough discrimination for bacterial identification, even for organisms that are not even considered at the time of array design. Using our experience in oligoarray design [1], this open strategy relies on a set of capture probes selected by an original bioinformatics strategy [2]. The strategy represents a balance between array complexity and the likelihood to reliably obtain discriminative biological signatures (i.e. a balance between cost and information). Such arrays have been validated *in silico* against published genomes for their capacity to: (i) identify the genetic background of the analyzed sample, (ii) compare biological signatures against reference database, (iii) discriminate samples at the species level, and (iv) provide enough discrimination power for strain genotyping and discrimination. Preliminary wet-lab results suggest that the approach is feasible. However, the range of GC contents in the tested organisms should be overcome (e.g. by appropriate hybridization buffers and/or analytical tools) to prevent major influence on fluorescence signals.

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DNA Microarray Technique for Detection and Identification of Viruses Causing Encephalitis and Hemorrhagic Fever

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The microarray technique allows simultaneous screening of a sample for many different nucleic acid fragments based on sequence similarities and therefore has a promising potential for virus detection and identification. We are developing microarray methods for viruses causing encephalitis and hemorrhagic fevers for which rapid and correct virus identification is important, making broad screening methods useful. Included are seven human pathogenic Flaviviruses, Filoviruses, Hantaviruses, Lassa virus, Rift Valley Fever virus and Crimean Congo Hemorrhagic Fever virus. The microarray technique also provides some advantages compared to other nucleic acid based methods regarding problems with unknown sequence variation in these RNA viruses.

We have combined amplification of viral nucleic acid in a sample with hybridization to a microarray with long probe fragments. Microarrays have been designed for Hantaviruses, Flaviviruses and a group of hemorrhagic fever viruses. Highly degenerated primers were used for amplification of sample material before hybridization to the Hantavirus and Flavivirus microarrays while random amplification was tested on the group of hemorrhagic fever viruses.

The capacity of long probes to tolerate mismatch in new strains was evaluated for Hantaviruses and Flaviviruses. For flaviviruses the microarray method demonstrated a lower limit of detection comparable to standard RT-PCRs and it was successfully tested on samples from Dengue infected patients. Random amplification allowed successful detection and identification of cellcultured Lassa virus, Crimean Congo Hemorrhagic Fever virus and Ebola Zaire virus.

The results are promising and the tests on clinical samples demonstrate the potential and usefulness of virus identification methods based on microarray technique.

Sensitive Detection of Pathogenic Bacteria Using Novel GyrB Based Microbial Diagnostic Microarray

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To determine the potential of the microarray-based method for the detection and identification of bacterial pathogens a pilot microarray was developed and evaluated. An adapted protocol for the sequence-specific end labelling of oligonucleotides was used in combination with a set of gyrase subunit B (*gyrB*) specific probes. The microarray was validated using a representative set of targeted strains as well as several related negative controls. Environmental applicability was tested with a set of environmental samples, both spiked and non-spiked with targeted strains.

We were able to show this diagnostic microarray to be capable of parallel detection of a broad range of pathogenic bacteria from complex environmental samples with high levels of both specificity and sensitivity. Specificity of the detection was further enhanced by the introduction of the novel competitive oligonucleotides concept. Sensitivity level was determined to be between 0.1 and 1%.

Identification of *Coxiella burnetii* with a novel Low-Cost-and-Density (LCD)-Microarray

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In our study we investigated the suitability of a new and fast “low cost and density”(LCD) DNA microarray for the detection of *Coxiella (C.) burnetii*, the causative agent of Q fever. Results were compared with a conventional and a LightCyclerHyprobe-PCR assay in terms of specificity and sensitivity.

The LCD-microarray has the format of a plastic slide-frame which contains eight identical microarrays in individually addressable hybridization fields, with the IS1111-region (repetitive element, up to 20 times) and the *adaA*-gene (recently proposed as a marker for acute Q fever disease). A non-fluorescent detection principle, based on the formation of a clearly visible substrate precipitate, is used. Analysis can be done with a simple transmission light scanning device or by pure eye only.

Eleven different *C. burnetii* strains derived from animals and humans with acute and chronic disease were analyzed. All strains showed in all three assays a positive reaction with both targets, with the exception of one chronic and one animal-derived strain that were negative for *adaA*. The sensitivity was determined to be up to 10 genomic copies/μl template for IS1111 and 100 copies for *adaA*. No unspecific reactions were seen with a DNA panel of related bacteria. The results of the realtime-and conventional PCR-method were similar to the LCD-Chip.

This new technique provides a very fast, economic, sensitive and specific detection of *Coxiella burnetii*. In addition, since no sophisticated lab equipment is needed, this method could be introduced into the field of clinical, experimental and epidemiological studies in animals and man.

Molecular Diversity and Antibiotic Susceptibility of *Bacillus Anthracis* Strains Causing Animal Death in Chad: Detection of New Phylogenetic Groups.

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Fifteen *Bacillus anthracis* strains have been isolated from carcasses of cattle in different regions of Chad and were analyzed by use of various markers. The standard multiple-locus variable-number tandem repeat analysis (MLVA) of eight markers was used to genotype the strains. All strains were identical using MLVA, and represent a novel genetic lineage, designated A β , in the previously described 'A' cluster. The analysis of three additional Random Amplified Polymorphic DNA (RAPD) markers was used to differentiate the Chadian strains into two unique genotypes (VII and VIII). Significantly, the Chadian strains were susceptible to 11 tested antibiotics. The MIC of ceftiofur, which is a 3rd generation cephalosporin restricted to animal use, was 8 μ g per ml. The susceptibility of *B. anthracis* to ceftiofur has never been tested before. The microarray-based analysis of the DNA revealed the presence of the β -lactamase genes *bla1* and *bla2* which are endogenous to *B. anthracis*, but which are not expressed. Besides these two β -lactamase genes, the strains were shown to be free of all tested antibiotic resistance genes. The low molecular diversity of Chadian *B. anthracis* genotypes and the absence of geographic clustering of the two genotypes, is likely a reflection of extensive long distance transhumance in the country. The molecular analysis of Chadian isolates suggests that this region contains a unique genetic lineage of *B. anthracis* that is represented by strains of two different genotypes.

SPR Based Biosensors

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Biosensors are powerful tools aimed at providing selective identification of toxic chemical compounds at ultratrace levels in industrial products, chemical substances, environmental samples (e.g., air, soil, and water) or biological molecules and systems (e.g., enzymes, antibodies/antigens, nucleic acids bacteria, virus, or tissue components) for biomedical diagnosis. One of the relatively new and most attractive optical biosensors is based on surface plasmon resonance (SPR) phenomenon which occurs when an incident beam of planar polarized light of a given wavelength strikes the surface (e.g., a glass slide) at a given angle through a prism coated usually with a thin gold layer. Bioligands are immobilized onto gold coated SPR slides, and the medium carrying the target molecules flow-through the channel where these slides are placed. The interaction on the surface can be monitored continuously by detecting the intensity change of reflected lights resulting from changes in the refractive index on the gold surface. The interaction is monitored in real time and the amount of bound ligand and rates of association and dissociation can be measured with high precision. SPR technique is applied to the measurements of ligand-receptor interactions, drug screening, DNA-hybridization, enzyme-substrate interactions, polyclonal antibody characterization, epitope mapping and label free immunoassays. SPR biosensors have various advantages including real-time measurement of molecular interactions without labeling (which is one of the main requirements of the most of the optical sensors, which makes the system much more complex and expensive) and by using a simple optical system for the device. Gold surface can easily be engineered using conventional patterning techniques to form arrays on the surface. With the recent improvements SPR based sensors show great potential to meet the need for rapid, sensitive and specific real-time detection of biomolecules.

Mimivirus giant particles incorporate a large fraction of anonymous and unique gene products endowed for antigenic properties

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Acantamoeba polyphaga mimivirus is the largest known virus both in particle size and genome complexity that was classified as the first member of the new *Mimiviridae* family. Recent clinical evidences raised the possibility that Mimivirus might be a human pathogen responsible for pneumonia. Its 1.2 Mb-genome encodes 911 proteins among which only 23% exhibit a convincing homology to proteins of known function and 39% of them do not exhibit a clear ($E < 10^{-5}$) sequence database match. As their number increases with each sequenced genome, the status of such species-specific putative genes termed ORFans became matter of controversy, with opinions ranging from considering them pieces of junk DNA to seeing them as encoding normally expressed functional proteins.

Here, the composition of purified isolated virions was analysed using a combined electrophoresis/mass spectrometry approach allowing identification of 114 proteins including 45 ORFans. These proteins are endowed for antigenic properties as demonstrated by both western-blot and immunogold staining. This work demonstrates that anonymous and unique genes constituting the majority of Mimivirus gene complement encode *bona fide* proteins that are likely to participate to well integrated processes.

Phage Display Technology

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Phage display is a technique that uses bacteria and phages to produce and select proteins with a desired trait. Here, a genetic material coding a peptide sequence is inserted into the bacteriophage genome and this fused gene is displayed as a peptide sequence on the protein coat of the phage. Phage display is a useful tool in combinatorial chemistry to create random molecules and then selectively screen them for desirable properties. It is most commonly used to produce affinity reagents such as scaffold proteins, antibodies, inhibitors. Displayed proteins may be used in protein arrays, separation, drug development, epitope mapping and protein-protein interactions. Phage display can be applied towards receptors (antibody, hormone, cell), organs, plastic surface, inorganics, etc. The M13 belongs to the filamentous bacteriophages. Phage library is prepared by insertion of peptides in a billions of diversity and phage pool is obtained with different phage particles having different peptide sequences. Phage display has a great potential in use for the detection of ligands towards biologically threatening agents. In an immuno-based detector device, probe molecule can be a peptide sequence selected by phage display recognizing the pathogenic agent, specifically. Recently, different peptide/antibody motifs (hepatitis C virus surface antigen, ebola and herpes simplex virus glycoprotein, bacillus spores, etc.) have been determined by this method. We aim to select peptides specific to *Mycobacterium Tuberculosis* and use these peptides as probes in the detection of the related bacteria.

Comparison of Antigenic Properties of *Rickettsia typhi* and *Proteus vulgaris* in Diagnosing Endemic Typhus

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Rickettsia typhi, a gram-negative obligatory intracellular bacterium, is the causative agent of endemic typhus. The zoonosis is maintained in rodents and transmitted to humans by the rat flea *Xenopsylla cheopis*. The disease is usually acute and mild, with a skin rash and fever. Although it has a worldwide distribution, it is frequently unrecognized, and documented cases are rarely reported, particularly in tropical countries. Due to the absence of specific and highly sensitive antigens, serological diagnosis of the infection is still based on the Weil-Felix test using *Proteus vulgaris* OX 19 as the antigen, and is often ambiguous. As antibodies produced against *R. typhi* cross-react with *P. vulgaris* OX 19, it has been anticipated that their lipopolysaccharides (LPSs) contain common immunoreactive epitopes in their O-specific chains. Our structural studies on the *R. typhi* LPS indicate that epitope(s) containing N-acetyl quinovosamine should be responsible for the common antigenicity of LPSs from *R.typhi*/typhus group rickettsiae and *P. vulgaris* OX19. The role of other sugar units in the epitope specificity has to be clarified in future.

Comparative Proteome Analysis of Fractions Enriched for Membrane-Associated Proteins from *Francisella Tularensis* Subsp. *Tularensis* and *F. Tularensis* subsp. *Holarctica* Strains

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The facultative intracellular pathogen *Francisella tularensis* is the causative agent of the serious infectious disease tularemia. Despite intensive research, the virulence factors and pathogenetic mechanisms remain largely unknown. In order to identify novel putative virulence factors we carried out a comparative proteome analysis of fractions enriched for membrane-associated proteins isolated from the highly virulent subspecies *tularensis* strain SCHU S4 and three representatives of subspecies *holarctica* of different virulence including the live vaccine strain. We identified six proteins uniquely expressed and four proteins expressed at significantly higher levels by SCHU S4 compared to the ssp. *holarctica* strains. Four other protein spots represented mass and charge variants and seven spots were charge variants of proteins occurring in the ssp. *holarctica* strains. The genes encoding proteins of particular interest were examined by sequencing in order to confirm and explain the findings of the proteome analysis. Our studies suggest that the subspecies *tularensis*-specific proteins represent novel potential virulence factors.

Preliminary Structural Studies on a Lipopolysaccharide from *Piscirickettsia salmonis*

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Piscirickettsia salmonis is the etiological agent of the salmonid rickettsial septicemia or piscirickettsiosis. The bacterium was the first Rickettsia-like organism recognized as a fish pathogen. *P. salmonis* infects a wide range of salmonid species and causes a systemic infection in fish targeting predominantly kidney, liver, spleen, intestine, brain, ovary and gills. Six *P. salmonis* immunoreactive antigens were observed, but only two of them show carbohydrate composition. The major carbohydrate antigen is presumed to be a lipopolysaccharide (LPS) but both its composition and structure are unknown. Using a hot phenol-water method an LPS was isolated that was shown to be of R (rough) type on SDS-PAGE. Compositional analyses revealed the presence of mannose (Man), glucose (Glc), galactose (Gal), L,D-heptose (Hep) and glucosamine (GlcN) in a molar ratio 3.0:4.8:3.0:1.0:0.4, respectively. Methylation linkage analysis indicated the presence of terminal Man and Glc and 6-linked Man and Glc (Gal) as the major sugar constituents. Minor amounts of 4-linked Man and Glc (Gal) and 2,3-disubstituted Hep were also detected.

Preliminary studies on the *P. salmonis* lipid A indicate that its primary structure could be similar to those of the classical enterobacterial lipids A. More detailed investigations are in progress.

An Outbreak of Q-fever in Bulgaria

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Q fever is a zoonotic disease caused by *Coxiella burnetii*, a rickettsial organism, pleomorphic coccobacillus with a Gram negative cell wall, which is distributed worldwide. Cattle, sheep, goats, domestic pets, wild rodents, birds and ticks are the primary reservoirs of this organism. *C. burnetii* is not associated with rickettsial disease in these animals, but increases abortion rate in goats and sheep. *C. burnetii* is excreted in milk, urine and faeces of infected animals. Humans are the only known host to develop illness as a result of infection.

In May - June 2004 an outbreak of Q-fever occurred in a small town, 100 km North of Sofia. Clinical signs of patients suggested atypical pneumonia. Initial analysis (microbiological, serological, and molecular) did not confirmed infection due to *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, influenza and other respiratory viruses. The outbreak involved 220 hospitalized patients. Serology tests confirmed *Coxiella burnetii* as etiological agent of the pneumonia. *Postfactum* PCR test confirmed *C. burnetii* in swab samples. Epidemiological investigations confirmed that the outbreak originated and spread from infected animals. Serum samples from 270 sheep, goats and cattle were tested. 74 of them were positive for *C. burnetii*.

In perspective, our intentions are to investigate Rolling Cycle Amplification (RCA) and Proximity Ligation amplification techniques for specific and sensitive detection of *Coxiella burnetii*.

Single Nucleotide Polymorphisms as Targets for Real-Time PCR and DNA-Array Based Identification of Bacteria

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While DNA sequences relatively large in size are typically used as targets for the molecular identification of bacteria at the genus or species level, discrimination at the sub-species level often rely on nucleotide sequence variability within short DNA stretches or even on Single Nucleotide Polymorphisms (SNPs). With a few number of properly chosen SNPs, it should be possible to discriminate bacteria belonging to different biovars, serovars or genomovars. The present work deals with the description and the validation of SNPs for the identification of some biosafety level 3 (BSL3) bacteria. The use of SNPs to discriminate *Burkholderia pseudomallei* from the closely related sub-species *B. mallei* is presented as well as SNPs able to discriminate 4 out of the 5 different biovars of *Brucella suis*. These SNPs were validated on a collection of strains by DNA sequencing and/or Real-Time PCR. The use of SNPs as targets for the DNA-array based identification and typing of BSL3 bacteria is currently being assessed.

Transcriptional Profiling as a Means to Diagnose Infectious Disease

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The possibility to use transcriptional profiling of the immune response in blood as a means to obtain early diagnosis of infectious diseases has been relatively little explored. We here demonstrate that we were able to identify a gene subset that may serve as diagnostic biomarkers for early diagnosis of the disease tularemia. The disease is caused by the highly contagious bacterium *Francisella tularensis*. We undertook an analysis of the transcriptional response in peripheral blood during the course of ulceroglandular tularemia by use of Affymetrix microarrays comprising 14,500 genes. Samples were obtained from 7 individuals at 5 occasions during two weeks after the first hospital visit and convalescent samples 3 months later. In total 265 genes were differentially expressed, 95 of which at more than one time point. The differential expression was verified with real-time quantitative PCR for 36 genes. The most prominent changes were noted in samples drawn on days 2-3 and a considerable proportion of the up-regulated genes appeared to represent an IFN- γ -induced response and also a pro-apoptotic response. Genes involved in the generation of innate and acquired immune responses were found to be down-regulated, presumably a pathogen-induced event. A logistic regression analysis revealed that 7 genes were good predictors of the early phase of tularemia. Besides identifying a potentially diagnostic gene subset, the identified genes may also help to understand the pathogenesis of the disease.

A Molecular Method for the Discrimination Between *Francisella tularensis* Subspecies and *Francisella*-like Endosymbionts

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Introduction

Environmental studies on the distribution of *Francisella* are hampered by the frequency of *Francisella*-like endosymbionts, that can mislead a positive result. The significance on this problem has recently been heightened by the classification of *F. tularensis* as a category A agent of bioterrorism. Since it is known that *F. tularensis* 16S rRNA gene PCR assays cross-react with *Francisella*-like endosymbionts of ixodid species, we have used the gene coding the 17kDa lipoprotein (TUL-4) of *F. tularensis* to discriminate between these bacteria.

Methods

A PCR targeting the TUL-4 gene of *Francisella* spp., followed by *reverse line blotting* (RLB) was set up in this study. For this, a generic probe for *Francisella* spp. as well as a common specific probe for *F. tularensis* species (subsp. *tularensis*, subsp. *holarctica* and subsp. *novicida*), and a specific probe for *F. tularensis*-like endosymbiont were designed. Available sequences from Genbank database and ClustalX and Mega3 software were used for the alignment and analysis. Environmental and clinical samples were studied. A synthetic DNA fragment was constructed following available sequences of *tul-4* from *Francisella* endosymbiont with overlapping primers up to 75 bp long in consecutive PCRs, to be used as a positive control.

Results and conclusions

The PCR showed a high sensitivity, and a specific identification by RLB was achieved for *F. tularensis* and *F. tularensis*-like endosymbiont. The use of this method allowed the simultaneous detection of multiple species in a single sample without the need of sequencing, and may be useful in laboratories that screen these species for diagnostic or surveillance purposes.

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Quality Assessment of Multiplex Oligonucleotide Microarrays for Detection of Select High Risk Pathogens

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A critical element in the event of an adverse biological incident or outbreak is the rapid detection of the biological agent, whether viral or bacterial. To this end we have developed a complete multi-pathogen oligonucleotide array for use in detection of both viral and bacterial pathogens from the high risk ‘Australia Group’ plus other select pathogens.

The array contains approximately 2000 oligonucleotide probes for around 130 different bacterial and viral pathogens plus some discriminatory probes for sub-strains. These include bioterrorism-associated agents such as *Yersinia pestis*, *Bacillus anthracis*, *Fransicella tularensis*, viruses i.e. Mopiea, Junin and Lassa amongst others. This multiplex array provides a rapid means by which we can identify unknown pathogens from clinical material with no prior knowledge of the infectious agent.

Using randomly amplified and labelled DNA samples we have assessed several sources of variation encountered including between slide printing batches, Cy3 labelling DNA samples, operators and days for routine hybridisations using various ‘high risk’ pathogens *Yersinia pestis*. This has highlighted the major causes of variation within the processing and analysis of samples using a microarray platform which have relevance to all methods utilising microarray analysis. Improvement in slide hybridisation consistency is being tested using automated systems to develop protocols for routine use in a clinical diagnostic setting.

A Clinical & Epidemiological Surveillance Program for Infectious Diseases with Bioterrorist Impact

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The first pilot study related to bioterrorism agents and surveillance systems for unusual situations, in Bucharest area, ran between 15 Nov 2004-15 Oct 2005. Two Romanian participant institutions (FVB and SVB) with 7 „work stations” (2 General Practitioner offices, 4 emergency rooms and 1 microbiology laboratory).

Objectives

General: development of a national system for identification, diagnosis and intervention in infection diseases with intentional character.

Specific: evaluation and connection of some national hospitals for infectious diseases, in order to obtain an organized response in case of unusual situations; awareness of the first line physicians, in order to improve the recognition of critical biological agents.

Method

- Randomised selection of 200 cases of acute infectious diseases, presented at FVB or SVB.
- Syndrom-based classification of patients: cutaneous, respiratory, digestive, neurologic or other infectious syndroms.
- High risk sub-syndroms definition for agents of group A, B and C (CDC classification for critical biological agents)
- Cuantification of cases caused by potential bioterrorist agents
- Clinical and epidemiological analyse based on biostatistical parameters.

Results

The sample includes: 42-cutaneous infectious syndroms, 47-respiratory infectious syndroms, 97-digestive infectious syndroms, 14-neurological infectious syndroms. Using our software algorithm we identified: 2 cases of group A agents (botulism); 51 cases of group B agents (A paratyphoid fever, salmonellosis, shigellosis); 0-group C cases.

Conclusion

Specific surveillance programs designed for the new microbiological threats, reemergence of some infectious diseases and bioterrorism events could be an important tool inside the public health system.

Key words: syndrom-based; critical biological agents; surveillance program.