



Domain Committee BMBS

(Biomedicine and Molecular Biosciences)

COST Action B28

***Array Technologies for BSL3 and
BSL4 Agents***

Annual Report

Period: from (27/5/2005) to (12/2007)

Action B28 Fact Sheet

Title

Array Technologies for BSL3 and BSL4 Pathogens

Contacts

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Details

MoU: 282/05
 CSO approval date: 15/03/2005
 End of Action: 29/05/2010

Start of Action: 30/05/2005
 Entry into force: 27/05/2005

Objectives

The main objective is to increase knowledge on BSL3 and BSL4 agents in order to support the development of more accurate diagnostics, vaccines and therapeutics, and to better understand epidemiology of these highly pathogenic microorganisms that potentially can be used as biological weapons.

Parties: list of countries and date of acceptance

Country	Date	Country	Date	Country	Date	Country	Date
Austria	06/07/2005	Belgium	01/06/2005	Bulgaria	20/05/2005	Czech Republic	30/06/2005
Denmark	26/05/2005	France	26/05/2005	Germany	25/05/2005	Greece	19/08/2005
Luxembourg	06/07/2005	Netherlands	01/06/2005	Portugal	13/12/2006	Romania	04/11/2005
Serbia	27/05/2005	Slovakia	06/07/2005	Spain	27/05/2005	Sweden	06/09/2005
Switzerland	06/07/2005	Turkey	05/01/2006	United Kingdom	21/06/2005		
Total:	19						

Intentions to accept: list of countries and date

Country	Date	Country	Date	Country	Date	Country	Date
Total:	0						

Other participants

None

Working Groups

None

Website

None

Management Committee

Chair	Vice Chair	DC Rapporteur
Professor Patrick BUTAYE General Bacteriology VAR Groeselenberg 99 Brussels BE pabut@var.fgov.be	Professor Rudolf TOMAN Rickettsial and Chlamidial Infections Institute of Virology Slovak Academy of Sciences Slovak Academy of Sciences Dubravska cesta 9 Bratislava SK virutoma@savba.sk	Professor Marieta COSTACHE Molecular Biology Center Faculty of Biology University of Bucharest 91-95 Spl. Independentei Bucharest RO costache@bio.bio.unibuc.ro

Austria

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Bulgaria

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Luxembourg

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Portugal

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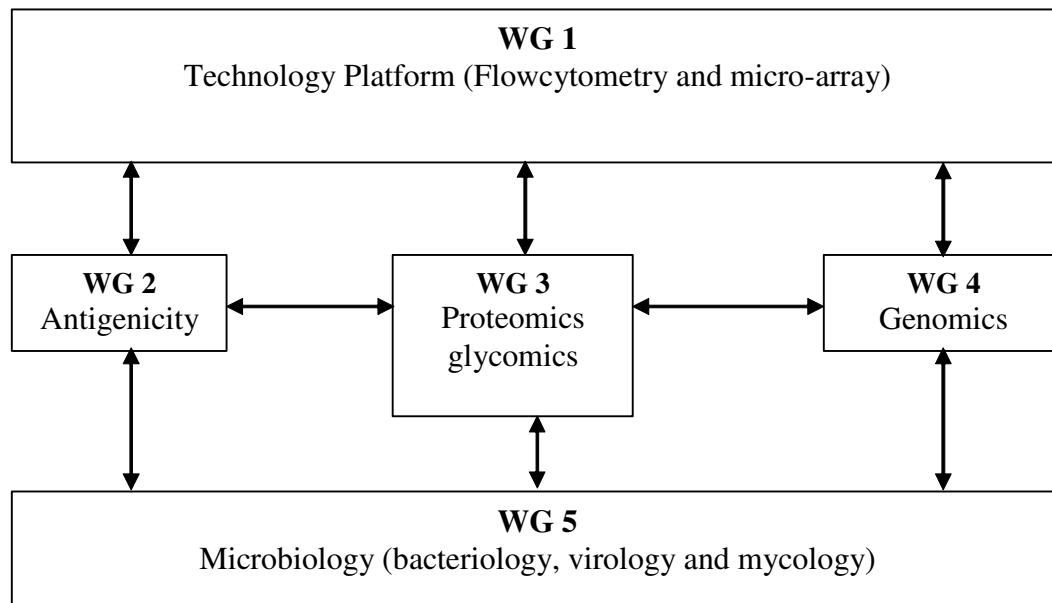
United Kingdom

Dr. Nigel SILMAN MC Delegate Centre for Emergency Preparedness & Response Health Protection Agency Porton Down Salisbury UK nigel.silman@hpa.org.uk

2. TECHNICAL DESCRIPTION AND IMPLEMENTATION

Describe the items of technical work, the mode of operation, possible subdivision in Working Groups, and how the secretarial services were organised (no more than 2 pages).

The technical work has been subdivided as shown in figure 1. 5 work packages have been created in order to cover all items of importance for the study of BSL 3 and BSL 4 agents.



Working group chairs have been identified during the first WG and second MC meeting. They will be in charge of the coordination of the work within the Working groups.

A subcommittee of 3 persons on the selection of STSM has been established during the first WG and second MC meeting.

3 PARTICIPATION AND COORDINATION

3.1 Management Committee

As shown above (Action fact sheet)

3.2 Participating Institutes

List: denomination and country without address

ARCS Seibersdorf
Austrian Agency for Health and Food Safety
Veterinary and agrochemical research center

Austria
Austria
Belgium

Scientific institute for public health	Belgium
UCL	Belgium
ULG	Belgium
National Center of Infections and Parasitic Diseases	Bulgaria
Bulgarian Association of Microbiologist	
Department of Microbiology	Bulgaria
Canadian Science Centre for Human and Animal Health	Canada
University of Defence	Czech Republic
University of Aarhus	Denmark
Statens Serum Institut	Denmark
INRA	France
AFSSA	France
Université de Marseille	France
Federal Institute for Risk Assessment (BFR)	Germany
Bundesforschungsinstitut für Tiergesundheit	Germany
Institut für Mikrobiologie der Bundeswehr	Germany
Universitätsklinikum Göttingen	Germany
Robert-Koch Institut	Germany
Helmholtz Centre for Infection Research	Germany
University of Ioannina	Greece
Laboratoire National de Santé	Luxemburg
Instituto Nacional de Saúde Dr. Ricardo Jorge	Portugal
Dr. Victor Babes Foundation	Romenia
Institute of Nuclear Science	Serbia and Montenegro
Slovak Academy of Sciences	Slovakia
Instituto de Salud Carlos III	Spain
Uppsala University	Sweden
Karolinska institutet	Sweden
Umea University	Sweden
University of Bern	Switzerland
University of Geneva	Switzerland
TNO	The Netherlands
Centraal Bureau voor Schimmelcultures	The Netherlands
RIVM	The Netherlands
WUR	The Netherlands
Hacettepe University	Turkey
University of Wales, Aberystwyth	UK
University of East London	UK
HPA	UK

3.3 Meetings of the Management Committee

Brussels, Belgium, 27/05/2005:

Bratislava, Slovak Republic, 22/11/2005

Antalya, 3 October, 2006

Brussels, June 7-8, 2007 (special meeting of the WG leaders with the DC)

Plovdiv, April 20-22, 2007

Vienna, December 12, 2007

3.4 Meetings of the Working Groups

Bratislava, Slovak Republic, 21-22/11/2005

Antalya, 1-3 October 2006

Plovdiv, April 20-22, 2007

Vienna, December 10-12, 2007

3.5 Short-term scientific missions

A core group of 3 persons has been assigned to evaluate the proposed STSMs. In 2007, 2 STSMs have been executed. Additionally a training school has been organised. The training dealt with working in a BSL3-BSL4 environment.

In the year 2007 two STSMs were accomplished.

First STSM (COST-STSM-B28-1321, COST-STSM-B28-02681) took place at the Institute of Molecular Pathology, Hradec Kralove, Czech Republic from October 21 to 27, 2007. Two young research workers Katarina Palkovicova (born Slaba) and Robert Ihnatko from the Laboratory for Diagnosis and Prevention of Rickettsial and Chlamydial Infections, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia were the persons who were approved by the COST ACTION B28 STSM Committee. The purpose of their STSM was

- to improve skills in the proteomic approaches involving 2-D electrophoresis
- to learn new methods of visualization, immunodetection and characterization of proteins with the aim to find and detect *Coxiella burnetii* immunoreactive proteins.

After completion of their STSM, both young researchers submitted a short scientific report to the COST ACTION B28 STSM Committee. The main results obtained were following:

- Efficiency of cell lysis using French press was highly enhanced in comparison with “traditional” preparation of *Coxiella burnetii* cell extracts based on chemical lysis.

- French press method is therefore more effective and more suitable for sample preparation and proteome analysis of *Coxiella burnetii* than the sample preparation previously established in the home laboratory

- Experimental skills were gained with more sensitive protein visualization methods on PVDF membranes from 2-D gels.

The second STSM (COST-STSM-B28-03144) took place at the Institute for Molecular Systems Biology, Zürich, Switzerland from October 1 to November 2, 2007. One young research worker Anetta Hartlova from the Centre of Advanced Studies, the Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic was the person who was approved by the COST ACTION B28 STSM Committee. The purpose of her STSM was

- to improve the lipid raft isolation method

- to learn new mass spectrometry tools for characterization of lipid rafts.

After completion of its STSM the young researcher submitted short scientific report to the COST ACTION B28 STSM Committee. The main results obtained were following:

- using CSC technology she was able to identify total plasma membrane proteins and lipid raft-associated proteins of J774.2 with a high specificity. Currently, there is no information regarding the J774.2 cell surface characterization. CSC technology enables to detect the cell surface molecules without antibodies.

- after IFN γ stimulation, two different phenotypes (non-stimulated vs. stimulated cells) were compared to each other.

In both STSMs, the COST ACTION B28 STSM Committee followed thoroughly all the research work that was planned and performed during these STSMs and could confirm that all the major goals have been achieved, and thus, it could recommend both reports to be accepted by the COST Office.

A training school was organised at the University of Göttingen, since it seemed difficult for laboratories to acquire permissions of people working for a short time under these specific conditions, a reason for why STSM were difficult to organise. There was a great interest in this training school, we even had to refuse people attending, since the maximum number of attendances of 10 was reached. Therefore also, an additional training school was planned for 2008. The Training school took 3 days, from may 22 to may 24, 2007. The morning sessions were theoretical sessions, the afternoon sessions were practical sessions of working in a BSL3 laboratory.

People learned on what to take care of under BSL3-4 conditions (e.g. disinfection, waste management). In the afternoon sessions, a practical training, using mock samples was performed. A certificate was handed over to all participants.

List of participants

Country	Institute	Name
1. Luxembourg	Laboratoire National de Santé	Mariette Ducatez Mariette.Ducatez@LNS.ETAT.LU
2. Luxembourg	Laboratoire National de Santé	Claude P. Muller claudemuller@LNS.ETAT.LU
3. Czech Republic	University of Defence, Facility of Military Health Service	Lukas Cerveny * cerveny@pmfhk.cz
4. Czech Republic	University of Defence, Facility of Military Health Service	Adela Strakova strakova@pmfhk.cz
5. Belgium	Centre for Applied Molecular Biology	Dr Leonid Irengé irengel@bcm.ucl.ac.be
6. Switzerland	Institute for Veterinary Bacteriology	Paola Pilo paola.pilo@vbi.unibe.ch
7. The Netherlands	TNO Defence, Security and Safety	Margo Molhoek desiree.vanderkleij@tno.nl
8. Serbia	Institute for Nuclear Sciences "Vinca"	Snezana Jovanovic-Cupic cupic@vin.bg.ac.yu

9. Bulgaria	National Center of Infectious and Parasitic Diseases, Microbiological department, National Reference Laboratory of Tuberculosis	Yuliana Asenova ulianaassenova@yahoo.com
10. Greece	University of Ioannina Department of Chemistry, Section of Organic Chemistry and Biochemistry	Sofia Zikou sofiazikou@yahoo.gr

Agenda

BSL3 / BSL4 Training School 22 – 24 May 2007

Institute of Virology, University Medicine Goettingen

Tuesday, 22 May 2007

Venue: Library, Institute of Virology, Kreuzbergring 57, Goettingen

	Lectures:	
09:15	Hazard Criteria and Categorisation of Microbes	Nigel Silman
09:45	BSL3 Lab Technical specifications	Frank Hufert
10:30	Protective Gear (including 15 min movie on laminar flow cabinets)	Nigel Silman Manfred Weidmann
11:15	Efficacy of Inactivation Procedures	Patrick Butaye
13:00	Practical course in the BSL3 Laboratory, Humboldt Allee 34 <ul style="list-style-type: none"> - Practical moving in and out of a BSL3 (including hand washing) - Dexterity at the laminar flow cabinet (mock dilution series of intensely coloured solutions in protective gear) - Inactivation of virus cell cultures test series setup - Cultivation of B. anthracis 	Supervisors: Frank Hufert, Manfred Weidmann, Patrick Butaye Nigel Silman Mandy Elschner
17:00	Anticipated end of the practical course	

Wednesday, 23 May 2007

Venue: Library, Institute of Virology, Kreuzbergring 57, Goettingen

09:00	Lectures: BSL4 Labs technical specifications BSL4 Labs in Europe	Ali Mirazimi
09:45	Learning from a history of lab accidents	Manfred Weidmann
11:00	Shipping BSL3 and BSL4 organisms/ IATA regulations UN regulations	Mandy Elschner
13:00	Practical course in the BSL3 Laboratory, Humboldt Allee 34 <ul style="list-style-type: none"> - Handling positive pressure masks - Dexterity at the laminar flow cabinet (mock dilution series of intensely coloured solutions, wearing positive pressure masks) - Inactivation of virus cell cultures test series - Interpretation of B. anthracis read out 	Supervisors: Frank Hufert, Manfred Weidmann, Patrick Butaye Nigel Silman Mandy Elschner
17:00	Anticipated end of the practical course	

Thursday, 24 May 2007

Venue: Library, Institute of Virology, Kreuzberggring 57, Goettingen

09:00	Lectures: Handling samples that arrive for diagnostic testing	Mandy Elschner Nigel Silmann
09:45	Bridging the gap between requirements of bio-containment and diagnostics	Manfred Weidmann
10:30	Waste Management	Patrick Butaye
11:15	Fumigation: Theory and Practice	Nigel Silman
13:00	Practical course in the BSL3 Laboratory, Humboldt Allee 34 <ul style="list-style-type: none"> - Inactivation of virus cell cultures test series read out - PCR read outs 	Supervisors: Frank Hufert, Manfred Weidmann, Patrick Butaye Nigel Silman Mandy Elschner
17:00	Anticipated end of the practical course	

4. SCIENTIFIC REPORT -RESULTS

4.1 Results of the MC meetings

First MC meeting

The first meeting of B28 was a MC meeting organised in Brussels at the COST secretariat on 30/5/2005. Ten MC members were present. An equal number of MC members was excused. At that time 12 countries assigned MC members and 5 signed the MoU.

Information on COST was distributed.

Patrick Butaye was elected as chair and Rudolf Toman as vice chair.

It was decided to have a first WG-MC meeting in Bratislava where the objectives and work programme will be better established and this at the WG level. A second aim of the meeting was to better get known with the activities of the participating institutions and universities in order to be able to establish collaborations.

Second MC Meeting

Election of an STSM panel:

The MC agreed on the following composure of the STSM Panel consisting of 3 people :

Rudolf Toman - vice chair of the action

Patricia Renesto-Audriffen

Maria Sarakellos

All the applications should be sent to Rudolf Toman as the leader of the panel who will treat the applications according to the COST regulations for STSMs

A B28 website will be constructed.

The following WG leaders were elected:

- A. WG 1
Jacques Schrenzel
- B. WG2
Claude P. Müller
- C. WG3
Jiri Stulik
- D. WG4
Stefan Panaiotov
- E. WG5
Mandy Elschner

The next WG-MC meeting was prepared and would be a combined WG-MC and International Conference meeting in Turkey. The conference subject would be innovative array technologies.

Third MC meeting

The attendance and organisation of the meeting was difficult therefore some actions were discussed and decided upon to improve that point:

The attendance rate of this meeting was not as high as last meeting and disappointed some partners of the COST action. Reasons for these low attendance rate were looked for and, the cost of the meeting, the combination with other meetings, and the recent bombing were possibly identified as causes.

Joachim Frey, who chaired a former COST Action had a similar experience, in which the second meeting was poorly attended. The action however evolved well and had a large attendance rate with many new participants during its evolution.

The discussion on attendances was ended with a positive note that the MC members and WG chairs will activate the WG members to participate to the meetings.

Patrick Butaye stressed on the fact that MC members should take their responsibilities and should attend the MC meetings as much as possible. If they are frequently unable, they can look for a replacing member for the MC (as there is for Belgium). Some countries should check with the website and their national representatives for their membership. Also, there is seemingly some degree of confusion cause by bad communication between COST and the national representatives. Also the website is not complete, compared to the list from the COST secretariat.

The Next meeting would be sooner following the last meeting to improve contact and to allow the participants that were absent in Antalya not to loose contact with the other participants. It was decided to organize a meeting in April 2007 in Bulgaria. Bulgarian participants and personally Dr. Panaiotov assumed the responsibility to contact, coordinate and organize the meeting.

Forth MC meeting

This meeting in Plovdiv was a great success. The MC meeting was together with the WG meetings and the 6th National Congress of the Bulgarian Society of Microbiology (BSM). Many visitors of the BSM meeting also visited the talks of the COST Action B28.

Unfortunately we did not register these persons so we cannot give exact numbers of visitors. During the MC meeting it was announced that there would be a training school organised by members of the WG5 group. This initiative was well approved.

During this meeting, a new procedure to accept new members to the working groups was accepted. Proposals from new member to become partner the COST Action B28 would be judged by the WG chair, the vice-chair and the chair. As such, new people would be quickly integrated into the action.

The initiative of WG5 to organise a strain collection. The template for entering strains was proposed and well accepted.

A new non-COST country was accepted as a partner of COST Action B28: South Africa.

The date and place for the next meeting was accepted: Vienna, December 10-12, 2007

Fifth MC meeting

The fifth MC meeting was held at Vienna, 12th of December.

This meeting was held in full optimism for the future. The WG meetings preceding the MC was well organised with good invited speakers and with a good attendance.

It was announced that a second BSL3-BSL4 training course will be organised in Göttingen in Germany from 27-29/ 2008. A training course organised by WG3, Jiri Stulik is planned. The course will be organised in Hradec Kralove, Czech Republic. Teachers and supervisors for the practical work have already been selected. The data is forseen somewhere in the period April-June 2008.

Also, WG3 is preparing a booklet, together with WG2 on antigenicity.

The strain collection database of WG5 is now on the website and will be updated when new submissions are given.

Two STSMs have been completed successfully. The training courses will contain also some STSMs. One STSM is planned between TNO and CODA. For the coming year, a renewed appeal for STSMs is launched.

A new meeting is forseen in September 2008 in Romania. The exact place and data will be announced in the coming months. As to improve contacts between the WG members and their chair, the following strategy for the meeting has been adapted: the WGs will be given a block of time that they can fill in. The WG chair, and not the local organiser is responsible for the agenda of the WG. The local organiser will first draft an agenda, with the invited speakers, the blocks for the WGs and the agenda of the MC (together with the chair). The local organiser will also compile the list of attendants.

4.2 Results of the WG meetings

4.2.1 Results of WG1 meetings

1)Name: Levente BODROSSY and Tanja Kostić

Contribution:

Design, validation and application of microbial diagnostic microarrays. Development and adaptation of novel techniques to improve sensitivity and specificity of detection via microbial diagnostic microarrays. Development of specific microarrays for *Salmonella* serotyping and for waterborne pathogens.

In 2007, we report further adaptation and development of the different microarray platforms for pathogen identification and characterization. In our group we have developed *Salmonella* serotyping and water pathogen microarrays. *Salmonella* serotyping array is based on the short oligonucleotide probes targeting *fliC*, *fljB*, *gyrB* and *atpD* marker genes and it distinguishes prevalent *Salmonella* serovars in Austria, the UK and Switzerland. Water pathogen microarray was developed using SSELO method and *gyrB* marker gene and is characterized by high specificity and high sensitivity of the detection. This microarray can reliably identify 25 of the most relevant bacterial water pathogens and indicator organisms and is so far the most comprehensive diagnostic tool for the assessment of the microbial water quality.

Our current work involves development of the *Salmonella* phage typing and *Listeria* serotyping microarrays based on the DArT approach.

Publications:

1. T.Kostić, A.Weilharter, A.Sessitsch and **L.Bodrossy**, High sensitivity, PCR-free detection of microorganisms and their functional genes using 70mer oligonucleotide diagnostic microarray. *Analytical Biochemistry*.346:333-335. 2005.
2. A.Loy and **L.Bodrossy**, Highly parallel microbial diagnostics using oligonucleotide microarrays. *Clinical Chimica Acta* 363(1-2):106-119. 2006.
3. **L.Bodrossy** and A.Loy, Oligonucleotide microarrays in microbial diagnostics. In: *Encyclopedia of Medical Genomics and Proteomics* (Editors: J. Fuchs and M. Podda). Online edition. DOI: 10.1081/E-EDGP-120041475. Taylor & Francis. 2006.
4. **L.Bodrossy**, N.Stralis-Pavese, M.Konrad-Köszler, A.Weilharter, T.Reichenauer, D.Schöfer, and A.Sessitsch. mRNA-based parallel detection of active methanotroph populations using a diagnostic microarray. *Applied and Environmental Microbiology*, 72 (2): 1672-1676. 2006.
5. A.Loy, M.W. Taylor, **L.Bodrossy** and M.Wagner, Applications of Nucleic Acid Microarrays in Soil Microbial Ecology. pp. 18-41. In: *Molecular Techniques for Soil and Rhizosphere Microorganisms* (Editors: JE Cooper, JR Rao). CABI Publishing, Wallingford, Oxfordshire, UK. 2006.
6. Sessitsch, E. Hackl, P. Wenzl, A. Kilian, T. Kostic, N. Stralis-Pavese, B. Tankouo Sandjong and **L. Bodrossy**. Diagnostic microbial microarrays in soil ecology. *New Phytologist*, 171:719-736. 2006.
7. **T.Kostić**, A.Weilharter, S.Rubino, G.Delogou, S.Uzzau, K.Rudi, A.Sessitsch and **L.Bodrossy**. A microbial diagnostic microarray technique for the sensitive detection and identification of pathogenic bacteria in a background of non-pathogens. *Analytical Biochemistry*, 360:244-254. 2007
8. B.Tankouo-Sandjong, A.Sessitsch, E.Liebana, C.Kornschober, F.Allerberger, H.Hächler and **L.Bodrossy**. MLST-v, multilocus sequence typing based on virulence genes, for molecular typing of *Salmonella enterica* subsp. *enterica* serovars. *Journal of Microbiological Methods*, 69:23-26.. 2007.
9. H.Wiesinger-Mayr, K.Vierlinger, R.Pichler, A.Kriegner, A.Hirschl, E.Presterl, **L.Bodrossy** and C. Noehammer. Accurate Identification of human pathogens isolated from blood using microarray hybridisation and signal pattern recognition. *BMC Microbiology*. 7(1):78. 2007.

Collaborations: in the field of pathogen detection:

- Knut Rudi, Matforsk, DK; Martin Wagner, Veterinary University, Vienna, Austria;
- Salvatore Rubino, University of Sassari, Italy;
- Alexander Loy, University of Vienna, Austria.
- AGES - Österreichische Agentur für Gesundheit und Ernährungssicherheit, Austria
- Defra – Department for Environment, Food and Rural Affairs, UK
- DarT P/L, Australia (Dr. Peter Wenzel)

2) **Name:** Jacques SCHRENZEL (Jacques.schrenzel@genomic.ch)

Contribution:

Design, development and validation of innovative microarray formats for detecting unknown or unusual pathogens. Two formats are currently under development: a non-cognate hybridization system (NCHS), i.e. an array with >8'000 probes to encompass all

combinations of two nucleotides on 13mers. The second format uses a hierarchical classification of 25mer probes to cover the 16S rDNA diversity. Each probe is then mapped to the node of the tree that it can determine.

For the year 2007, we report the development of two novel microarray design approaches for the characterization of genetic signatures, and their application for diagnostic usage, to three *B. anthracis* strains. The first microarray strategy is based on a hierarchical probe design where each of the ~10'000 probes matches a different level of the 16S phylogenetic tree (domain, phylum, class, order, family, genus, species). This array covers the large majority of bacterial pathogenic, commensal and environmental strains. The second approach relies on a probe design scheme that allows targeting multiple organisms without having prior knowledge of their genomic content. This strategy named Non Cognate Hybridization System (NCHS) is based on 13-mer probes providing all combinations of only two nucleotides. This oligonucleotide length provides an optimal ratio between the number of required NCHS probes and the number of probes actually able to discriminate different targets. Hybridization of 3 different *B. anthracis* strains using the aforementioned strategies yielded to a probe pattern common to these strains. Both approaches should prove useful for the rapid diagnosis and characterization of microorganisms potentially involved in biotreats.

Publications:

Under revision in Applied Environmental Microbiology: A Novel Microarray Design Strategy to Study Complex Bacterial Communities, by Antoine Huyghe, Patrice Francois, Yvan Charbonnier, Manuela Tangomo-Bento, Eve-Julie Bonetti, Bruce J. Paster, Ignacio Bolivar, Denise Baratti-Mayer, Didier Pittet, and Jacques Schrenzel.

Collaborations:

- with the group of Patricia RENESTO (Marseille, France) for pathogen annotation and microarray analyses.
- with the group of Pierre WATTIAU and Patrick BUTAYE (Brussels, Belgium) for implementation and validation of a dedicated low-density array (ClonDiag).
- with Manfred Weidmann (Freiburg, Germany, Jean-Luc Gala (Brussels, Belgium) and Karen Kempel (Geneva, Switzerland) grant application in production for development of selective amplification methods for synthesis of pathogen-specific targets from clinical material

3) Name: Karl Walravens (Karl.walravens@var.fgov.be)

Contribution:

- Development and validation of a bead array for the multiplex detection of zoonotic pathogens by the help of a FACS. Validation of bacterial strain typing by the help of Monoclonal antibodies and FACS analysis.

In 2007, we report the development of typing methods based on the use of monoclonal antibodies directed against *Brucella* biovar specific antigens. The validation of this typing method was performed on almost 100 strains of *Brucella suis* from different biovars. The results obtained were in concordance with classical (Biochemical) typing with the exception of *Brucella suis* biovar 3 originating from Croatia that were identified as *B. suis* biovar 1. However other genetic based typing methods like VNTR, MLST and AMOS PCR also characterize these two strains as biovar 1 and not 3 of *Brucella suis*. We also

implemented the VNTR typing method (MLVA) on *Brucella suis* and *Brucella abortus* from the CODA-CERVA strain collection in order to further characterize the discriminatory capacity and robustness of different sets of minisatellite loci for the typing of these species of *Brucella*. The work is under way.

We also participate to the validation of a multiplex PCR typing assay developed by the group of Ignacio Lopez-Goni from the University of Navara.

Collaborations:

- Martien Broekhuizen/Jasper Kieboom, exchange of strains.
- Ignacio Lopez-Goni University of Navara. Brucella typing via a multiplex typing assay.
- In development: Rudolph Toman, development of monoclonal antibodies against different bacteria.
- Martien Broekhuizen, members of the former COST action 845 (*Brucella* typing work).
- Martien Broekhuizen/Jasper Kieboom, exchange of strains.
- In development: Rudolph Toman, development of monoclonal antibodies against different bacteria.

4) Name: Jasper Kieboom (jasper.kieboom@tno.nl), replacing Martien BROEKHUIJSEN (martien.broekhuijsen@tno.nl)

Contribution:

Design and development of microarrays for 1) genomic comparison of *Brucella* strains, 2) multipathogen identification.

In 2007, we report the development of a microarray for genotyping of *Brucella*. From initial experiments some unique oligo-DNA sequences were identified that could prove useful for the rapid diagnosis and characterization of microorganisms potentially involved in biothreats.

Publications: in preparation

Collaborations:

- with the group of Pierre WATTIAU and Patrick BUTAYE (CODA-CERVA, Brussels, Belgium) for microarray analysis of Brucella;
- with Manfred WEIDMANN (university Göttingen, Germany) and Nigel SILMAN (HPA Porton Down, UK) for development of multipathogen microarray analysis.

5) Name: Ulrich Nübel (nuebelu@rki.de)

Contribution:

Bacterial genotyping on the basis of DNA microarrays: diagnostic detection of taxonomic markers, virulence associated genes, and antibiotic resistance determinants; single nucleotide polymorphisms; comparative genome hybridizations

Publications:

Antwerpen et al., DNA microarray for detection of antibiotic resistance determinants in *Bacillus anthracis* and closely related *Bacillus cereus*, Mol. Cell. Probes, in press
Additional manuscripts in preparation

Collaborations:

- Patricia RENESTO / Didier RAOULT (Marseille, France), microarray diagnostics of *Rickettsia* spp.
- Rudolf TOMAN (Bratislava, Slovakia), microarray diagnostics of *Rickettsia* spp.

6) Name: Gareth W GRIFFITH and Hazel M. DAVEY (gwg@aber.ac.uk; hlr@aber.ac.uk)

Contribution:

Use of flow cytometry for the detection of airborne bioaerosols, including biowarfare agents and other pathogenic microbes. Use of fluorescently-tagged antibodies allows the specific detection of target propagules in a background of other bioaerosol materials, in association with evolutionary computing methods for defining gating parameters. The system is being tested with spores of / *Aspergillus fumigatus* in the first instance but it is generic in its capability for detection. Developing methods for rapid determination of microbial viability and monitoring the effectiveness of antibiotic therapy.

Publications:

Assunção, P., Antunes, N.T., de la Fe, C., Rosales, R.S., Poveda J.B and Davey, H.M. (2006) Flow cytometric determination of the effect of antibacterial agents on *M. agalactiae*, *M. putrefaciens*, *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* large colony-type. Antimicrobial Agents and Chemotherapy 50: 2845-2849.

Collaborations:

With the group of Erhan Piskin (Ankara, Turkey) for the development of specific antibodies.

7) Name: Michael MULVEY (Michael_Mulvey@phac-aspc.gc.ca), Replaced by Steven Jones (Steven_Jones@phac-aspc.gc.ca)

Contribution: Development of standardized molecular forensic methodologies for the typing of *B. anthracis*, *F. tularensis*, and *Y. pestis*. Development of improved methodologies, such as single nucleotide polymorphisms (SNPs) and single nucleotide repeats (SNRs) for typing the above listed organisms. The use of DNA microarrays for comparative genomic and expression analysis of *B. anthracis* genomes.

Publications:

Stratilo CW, C. Lewis, L. Bryden, M. R. Mulvey, D. Bader. 2006. Identification of *Bacillus anthracis* isolates using single nucleotide repeat analysis (SNRA). Journal of Clinical Microbiology, 44:777-782.

Collaborations:

Dr. Silman (United Kingdom) and Mats Forsman (Sweden) for standardizing molecular typing methodologies.

8) Name: Ulf LANDEGREN (ulf.landegren@genpat.uu.se).

Contribution: Participation in the COST Action B28, “Array Technologies for BSL3 and BSL4 Pathogens” as representative of the EU Integrated Project MolTools that is coordinated by Ulf Landegren. The MolTools project aims to develop array-based tools for analysis at levels of DNA, RNA, protein, and also viable cells. We are pleased to interact with this COST Action to disseminate information arising in our project that could be relevant for pathogen

detection. Several recent developments in MolTools could be of interest for the COST Action, and three are mentioned here:

The group of Jörg Hoheisel in Heidelberg have established arrays with mirror-image L-form oligonucleotides, capable of hybridizing to corresponding tags on probes, but exhibiting no tendency to hybridize to DNA sequences representing biological sequences (Hauser et al. Nucl Acids Res 34: 5101-5111, 2006).

Another tag-array approach developed in Uppsala is in preparation, and involves a dual-tag recognition step on arrays, which ensures both high specificity and sensitivity because of inbuilt amplification steps (Ericsson et al. in preparation). Tag array approaches could be valuable for detection of pathogens since they allow solution-phase probes to detect and amplify signals, before sorting on arrays.

Finally, also in Uppsala, the so-called proximity ligation technique has been applied for detection of viral particles and bacteria (Gustafsdottir et al. Clin Chem 52: 1152-1160, 2006). The detection sensitivities observed were far superior to that of ELISA and similar to real-time PCR of the nucleic acids of the infectious agents, and sample preparation steps were minimal.

More information about the project can be found at www.moltools.org.

Publications:

Gustafsdottir SM, Nordengrahn A, Fredriksson S, Wallgren P, Rivera E, Schallmeiner E, Merza M, Landegren U. Detection of individual microbial pathogens by proximity ligation. Clinical Chemistry 52: 1152-1160 (2006).

9) Name: Jean-Luc gala (gala@lbcm.ucl.ac.be)

Contribution: design, development and validation of microarray for typing bacteria. Low high density microarrays have been used to identify bacterial species. Beads technology is now being developed for same purposes. Alternatively, the CMOS chips are being investigated for the detection of SNP.

In 2007 the following activities in the framework of COST Action B28 have been performed:

1. Efforts have been pursued to complete the microarray platform (with low or high density arrays, preformatted or customized arrays) including Affimetrix, Nimblegene, Agilent, Pamgene and Eppendorf platforms. Aspects involving bioinformatics and biostatics are carefully considered within a consortium and supported with a French spin off. The goal is to improve data normalization and standardisation across the various platforms to achieve reliable clinical diagnosis in the 2008.
2. Development regarding lab on chips based on nanotools are also pursued within a Belgian consortium. The data are still experimental and not yet clinically applicable.
3. Improvement of the pre-analytical processing of clinical samples in order to achieve a better recovery of DNA and RN from those biological samples. a special effort has been made on difficult samples (such as faeces, tissue and bone samples).
4. Development of bioinformatic stool to improve the detection of specific bacterial DNA in a bacterial or human background. This project is coordinated with a French team from the Hôpitaux de l'Assistance Publique. This part of the project will be used in a collaborative project inside the COST.
5. Development of fast PCR diagnostic tool by real-time PCR for a panel of BSL3 and BSL4 agents

Publications on microarray:

1. S. Hamels, J.L. Gala, S. Dufour, P. Vannuffel, N. Zammateo, J. Remacle. Consensus PCR and microarray for diagnosis of the genus *Staphylococcus*, species, and methicillin resistance. *Biotechniques* 31 (2001) 1364-1366,1368,1370-1372.
2. Lecouvet, L.M. Irengé, B. Vandercam, A. Nzeuseu, S. Hamels, J.L. Gala*. The etiologic diagnosis of infectious discitis is improved by amplification-based DNA analysis. *Arthritis & Rheumatism*, 2004; 9: 2985-94.
3. Herbert Tomaso, Daniela Jacob, Meike Eickhoff, Holger C. Scholz¹, Sascha A Dahouk, Mireille M. Kattar, Udo Reischl, Helga Plicka, Jaran O. Strand, Simo Nikkari, Pirjo Matero, Christian Beuret, Florigio Lista, Jean-Luc Gala, Hermann Brol, Bernd Appel, Ricela E. Sellek Cano, Maria del Carmen Ybarra de Villavicencio, Martien Broekhuijsen, Alexander Indra, Roger Petersen, Heinrich Neubauer. A multicenter evaluation of real-time PCR assays for the identification of *Yersinia pestis*. Submitted, 2007
4. Leonid M. Irengé, Karl Walravens, Marc Govaerts, Jacques Godfroid, Valérie Rosseels, Kris Huygen, Jean-Luc Gala. Rapid detection and specific identification *M. avium* subsp. *paratuberculosis* in fecal samples from experimentally infected, subclinically affected cattle by a triplex real-time PCR: comparison with conventional methods. Submitted, 2007.
5. Bernard Vandercam, Sabine Jeumont, Olivier Cornu, Jean-Cyr Yombi, Frédéric Lecouvet, Léonid M. Irengé, Jean-Luc Gala. Amplification-Based DNA Analysis in the Diagnosis of Prosthetic Joint Infection. *Journal of Molecular Diagnosis* Second revision 2007
6. Léonid M. Irengé, Jean-François Durant, Xavier Banse, Michel Lambert, Frédéric Lecouvet, and Jean-Luc Gala. Real-time PCR for identification of causative agents of infectious spondylodiscitis using molecular methods : an update. In preparation.

Collaborations:

- with Eppendorf Array Technology for microarray analyses.
- With Manfred Weidman (Freiburg, Germany) on the diagnosis of EBOLA and monkey pox on the field
- with Manfred Weidman (Freiburg, Germany), Jacques Schrenzel (Geneva, Switzerland Tania Kostic (Austria, Vienna) and Karen Kempse (Proton Down, UK): grant application in production for development of selective amplification methods for synthesis of pathogen-specific targets from clinical material
- With Karl Walravens on the validation of specific and sensitive real-time assay on difficult clinical samples (faecal samples in an experimental model of paratuberculosis);

10) Name: Erhan Pişkin (piskin@hacettepe.edu.tr)

Contribution: design, development and validation of innovative microarray formats based on surface plasmon resonance and elipsometer for detecting different type of pathogenic bacteria. These studies were initiated by using different species of mycobacterium which causes tuberculosis. This is based on hybridization on array surfaces.

For 2007 we report the development a nucleic acid-sensor based on Surface Plasmon Resonance (SPR) for label free detection of *Mycobacterium tuberculosis* (MTB). A single-strand oligonucleotide (ssDNA) carrying a thiol-end group which is complementary of the

target characteristic sequence of MTB was used as ligand (“probe”). MTB probe molecules were immobilized onto the gold surface via self-assembled monolayers (SAM) technique. In order to control the surface configuration of the probe DNA molecules, in other terms to orient it vertically on the surface, a modulator spacer molecule 6-mercapto-1-hexanol (MCH) was co-immobilized. Effect of temperature on the hybridization of MTB probe DNA molecules was also examined. Results showed that hybridization was increased with temperature at 25°C and 37°C compare to low temperature (i.e., 10°C). However, relative refractive index response at 45°C was decreased due to secondary structure of DNA molecules. In addition to these, reversible using and storage stability of prepared MTB sensors were investigated in this study. Reversible using data were shown that even after 4th regeneration; sensor response was above noise level, $57 \pm 4.1 \times 10^{-6}$ for MTB sensor. After 30 days ageing, sensor response 23 % decrease for MTB sensor.

We report the oriented immobilization of IgG molecules on the silicon surfaces. A multiple-step procedure was applied for oriented immobilization of IgG in this study. After hydroxylation of the Si(001) surfaces, 3-glycidoxypropyltrimethoxysilane (GPTS) molecules were self-assembled onto these substrates. Dipping time and GPTS concentration were found to be effected by on both layer thicknesses and water-contact angles. 2,2'-(ethylenedioxy)diethylamine (EDA) molecules were then covalently attached to the silicon surface with GPTS molecules. Imaging ellipsometry and atomic force microscopy (AFM) images exhibited aggregate formation at this step. Protein-A molecules were bound to the free amino groups of EDA molecules on the substrate surface, especially onto the aggregates by using a carbodiimide (i.e., EDAC) as the activating agent. We were able to immobilize IgG molecules in an oriented form onto the protein-A attached surfaces, especially in the regions, where EDA aggregates are located.

We report the investigation of a novel DNA biosensor based on ellipsometry. The effects of dipping time, solution concentration and solvent types on the formation of self-assembled monolayers (SAMs) with 3-mercaptopropyltrimethoxysilane (MPTS) molecules on the Si(001) surfaces were studied. Ellipsometric measurements showed that monolayers with a thickness of about 0.73 nm were formed when the dipping time is about 1 h, while more profound agglomerations were observed for longer time periods and MPTS solutions with higher concentrations. Monolayers were formed with solvents having larger dielectric constants. Contact angle measurements were in good correlation with the ellipsometric data. 5'-Thiolated oligodeoxynucleotides (ODNs) probes were immobilized onto these thiol-terminated SAMs by disulfide bond formation. The thickness of the ODN-layer on the MPTS modified surfaces reached almost a constant value of about 2.5 nm in 1 h. Target ODNs were detected by monitoring hybridization onto the surfaces by ellipsometry. The analytical signal (the delta (Δ) value) measured was correlated with the target ODNs concentration.

We report the investigation of the effects of dipping time, solution concentration and solvent type on the formation of self-assembled monolayers with aminosiloxane molecules (i.e., N-(3-trimethoxysilylpropyl)diethylenetriamine (TPDA)) on the Si(001) surface for possible array applications. Studies performed with an ellipsometry showed that monolayers with a thickness of about 1.2 nm were formed when the dipping time is about 2 h, while multilayer were observed for longer time periods. The effect of the TPDA concentration on the thickness of the deposited layer was not very profound; however, the contact angle data exhibit importance of concentration on the surface coverage. The type of the solvent used in the formation of the monolayers was found an important parameter. Monolayers were formed with solvent having larger dielectric constants. Relatively thick multilayer was observed when benzene was used as the solvent, due to its quite low dielectric constant (hydrophobicity).

We report the development of a thermo-responsive (“smart”) biosensor (array) platform. Poly(*N*-isopropylacrylamide) (poly(NIPA)) carrying two different functional groups (-SH and

-COOH) at two ends was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization. Self-assembled monolayers (SAMs) with amino terminated groups on the Si(001) surfaces were prepared using 3-aminopropyltrimethoxysilane (APTS). Poly(NIPA) molecules were then covalently attached to the silicon surfaces via APTS molecules. Afterwards, 5'-thiolated oligodeoxynucleotide-probes were immobilized onto these thiol-terminated poly(NIPA) layers on the surface by disulfide bond formation. An ellipsometer was used for detection (by hybridization) of the target oligos (the complementary of the probe oligos) within the aqueous media at two different temperatures, 25°C and 45°C, which are below and above the "Lower Critical Solution Temperature" (LCST) of poly(NIPA), respectively. Hybridization at low temperatures was significantly higher than those observed at higher temperatures. No response (no hybridization) was monitored when the target is a non-complementary oligo sequence. These preliminary studies demonstrated that this approach can be used to switch off and on the surface reactions on smart surface by using an external stimulus (temperature in this case).

We report the development of a nucleic acid-sensor based on Surface Plasmon Resonance (SPR) for label free detection of Influenza A and B virus. A single-strand oligonucleotide (ssDNA) carrying a thiol-end group which is complementary of the target characteristic sequence of Influenza A and B virus (i.e., for Influenza A 5'SH-(CH₂)₆-CTG CAG CGT AGA CGC TTT GTC CAA AAT G-3' and for Influenza B 5'SH-(CH₂)₆-ACC TTC GGC AAA AGC TTC AAT ACT CCA-3') was used as ligand ("probe"). This specially designed probe was immobilized onto the gold coated SPR slides via the thiol-end groups. In order to control the surface configuration of the probe, in other terms to orient it vertically on the surface, a modulator spacer molecule mercaptohexanol (MCH) was co-immobilized. The interaction (hybridization) between the immobilized probes on the SPR slides with the target ssDNA in the aqueous solution was followed successfully. Optical thicknesses of the hybridized surfaces were monitored using imaging ellipsometer to predict the relative quantity of overlayer materials on the chip surface. There was a quite significant effect of the modulator molecule. A novel assay for the optical detection of DNA sequences related to the Influenza A and B viruses, using surface plasmon resonance (SPR) technology were presented. Thiolated probes were attached covalently onto the gold sensor chip using self-assembled monolayer (SAM) technology. Various factors, affecting the probe immobilization, target hybridization were optimized to maximize the sensitivity and reduce the assay time and main analytical parameters, i.e. selectivity, sensitivity, reproducibility, analysis time, etc. were examined.

We report an immobilization procedure based on direct coupling of thiol-derivatised oligonucleotide probes to gold sensor surface has been used for detection of a pathogenic microorganism, *Mycobacterium gordonae* which is a non-tuberculosis (NTB) species of Mycobacteria family. In particular, synthesized target which is complementary of selected probe has been applied and biomolecular interaction analysis has been monitored by a label-free optical detection system: surface plasmon resonance (SPR) sensor. In the first part of this study, optimization of SPR chip surface was investigated. For this purpose, gold coated chip surfaces were cleaned by various recipes to get effective surface interaction. In order to get a well-oriented SAMs of both thiol-modified oligonucleotide probes and surface blocking agents (mercaptohexanol, MCH) on the gold surface, main parameters such as concentration, duration of treatment and type/concentration of buffer solutions have been studied. Performance of biosensor has been evaluated against to traceability of hybridization reaction between probe and target pair by using SPR sensor. For this purpose, targets having a sequence of 5'-GA CAG CAC CCG AGG GTG-3' were hybridized with probes having a sequence of 5'-HS-(CH₂)₆-(T)₁₅ CAC CCT CGG GTG CTG TC-3'. As a result of immobilization and hybridization steps, obtained organic layer formations were investigated

by using nulling-ellipsometry. Selectivity of sensor to specific target was also evaluated against to *Mycobacterium tuberculosis* targets. Insignificant sensor response was detected for control and non-specific target binding. Engineered sensor surface in this work for the detection of *Mycobacterium Gordonae* exhibits good sensitivity and specificity.

We report a stepwise formation approach to improve ellipsometric biosensor response. It is known that final formations of the molecules on the solid surfaces play an important role for further applications such as biosensor chip technologies. For this aim, we have firstly investigated the effects of dipping time and solution concentration on formation of self-assembled monolayers (SAMs) with 3-aminopropyltrimethoxysilane (APTS) molecules on the glass (BK7) surfaces. Surfaces were characterized by ellipsometry and water contact angle. Sensor chip surface were modified by gold nano-particles (AuNPs) having a nominal size of 40 nm on which mercaptoundecanoic acid (MUA) immobilized via thiol groups. MUA immobilization conditions were optimized by localized plasmon resonance (LPR) phenomena. Nulling/imaging ellipsometry system with flow cell arrangement was used to monitor bio-interactions on the sensor chip surface. Ellipsometric data in terms of relative change in delta (Δ) was recorded as sensor signal during the interaction of serum albumin from bovine (BSA) and carboxyl groups over the surface by means of carbodiimide activation. In order to evaluate the influence of the AuNPs to sensor response, another sensor chip for the comparison was also prepared with 3-mercaptopropyltrimethoxysilane (MPTS) and MUA by means of carbodiimide activation. A significant sensor response enhancement was observed from the surface having AuNPs compared to other.

Publications:

1. under preparation: Detection of *Mycobacterium tuberculosis* using DNA Based Surface Plasmon Resonance Biosensor, by Memed Duman, M. Oğuzhan Çağlayan, Gökhan Demirel, Erhan Pişkin.
2. Gökhan Demirel, Mustafa Oğuzhan Çağlayan, Bora Garipcan, Memed Duman, Erhan Pişkin. Oriented Immobilization of IgG on Hydroxylated Si(001) Surfaces via Protein-A by a Multiple-Step Process Based on a Self-Assembly Approach. *Journal of Material Science*, (2007), 42, 9402-9408.
3. under revision in *Surface Science*, A novel DNA biosensor based on ellipsometry, by Gökhan Demirel, Mustafa Oğuzhan Çağlayan, Bora Garipcan, Erhan Pişkin.
4. Gökhan Demirel, Mustafa Oğuzhan Çağlayan, Bora Garipcan, Memed Duman, Erhan Pişkin. Formation and Organization of Amino Terminated Self-Assembled Layers on Si(001) Surface, *Nanoscale Research Letters*, (2007), 2, 350-354.
5. under revision in *Langmuir*. Smart Poly(*N*-isopropylacrylamide) Layers on Self-Assembled Monolayers on Silicon Wafers As Smart DNA-Sensors (Array) Platforms, by Gökhan Demirel, Zakir Rzaev, Süleyman Patir, Erhan Pişkin
6. under preparation: A novel approach for the detection of influenza a and b virus by a surface plasmon resonance biosensor based on oligonucleotide interactions, by, Memed Duman, Mustafa Oguzhan Caglayan, Mehmet Ozsoz, Erhan Pişkin.
7. under preparation: Surface Plasmon Resonance Based Oligonucleotide Chip for the Detection of *Mycobacterium gordonae*, by, Memed Duman, Mustafa Oguzhan Caglayan and Erhan Pişkin.
8. under preparation: Stepwise formation approach to improve ellipsometric biosensor response, by, Mustafa Oğuzhan Çağlayan, Gökhan Demirel, Filiz Sayar, Burcu Otman, Burcu Çelen, Erhan Pişkin

Collaborations:

- Submission of FP6 programme on SPR for mycobacteria with partners from COST Action B28
- with the group of Mehmet OZSOZ (Ege Universitesi, İzmir, Türkiye)

11) Name: Dimitrios FRANGOULIDIS (DimitriosFrangoulidis@Bundeswehr.org)

Contribution: design, development and validation of a novel Low-Cost-and-Density (LCD)-Microarray for the detecting of *Coxiella burnetii* and other unusual pathogens. One prototype is finished and the sensitivity was determined to be up to 10 genomic copies/µl template for IS1111 and 100 copies for *adaA*. Array modifications according to the design of the internal control are just planned.

Publications:

The publication of the Cox-Chip is in progress.

Collaborations:

- with the group of Raquel ESCUDERO (Madrid, Spain) for strain exchange.
- with the group of Rudolf TOMAN (Bratislava, Slovak Republic) for further testing and validation of the Cox-LCD-Chip (Chipron) including further evaluation with different materials and strains.

12) Name: Karen Kempsell (karen.kempsell@hpa.org.uk)

Contribution:

We report (1) continued validation of our pan-pathogen array for select 'high-risk' pathogens; We have now validated our diagnostic array for a number of bacterial and viral pathogen groups including *Yersinia*, *Bulkholderia*, *Brucella*, *Bacillus*, *Francisella*, *Haemophilus*, *Listeria*, *Streptococcus*, *Staphylococcus* and *Neisseria* species, Crimean-Congo Haemorrhagic, Ebola and West Nile fever viruses. Work is also in progress to select pathogen-specific discriminatory probes for further assay development. (2) Continued development of robust methodologies for the use of microarrays for diagnostic use; we have begun investigating alternative protocols to improve the specificity of our hybridizations. This is of great importance for developing the technology for routine diagnostic use using clinical specimens (3) conclusions from our multi-operator microarray quality assessment; we investigated the sources of technical variability which can arise during microarray technological procedures. The study delineated potential sources of experimental and technical error and investigated remedial measures which can be taken to reduced experimental error.

Publications:

3 publications currently in preparation

Collaborations:

- With Dimitrios Frangoulidis at the Bundeswehr, six laboratory scientists visited the Bundeswehr laboratory in August 2007 for training in manipulation and culture of various pathogens including *Coxiella* and *Rickettsia* species.
- With Manfred Weidmann (Freiburg, Germany), Jean-Luc Gala (Brussels, Belgium) and Jacques Schrenzel (Geneva, Switzerland) grant application in production for development of selective amplification methods for synthesis of pathogen-specific targets from clinical material
- Martien BroekHuijsen, Jasper Kieboom; ongoing collaboration with respect to bio-threat diagnostic microarray development

13) Name: Henrik Nordström and Ake Lundkvist

Contribution:

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

A microarray system for detection and identification of viruses causing severe encephalitis and hemorrhagic fever is developed. The system is based on random or semi-random amplification of viral nucleic acid from a sample followed by hybridisation to a microarray. The microarray consists of 500-basepair PCR probe fragments corresponding to different parts of the viral genomes. The direct application of the assay is for unknown clinical samples from patients showing symptoms of severe encephalitis or hemorrhagic fever. The long probes in combination with the broad amplification provides a good possibility to identify new strains of known viruses.

Publications: in preparation

Collaborations:

Peter Nilsson at the Microarray facility at the Royal Institute of Technology in Stockholm.

14) Name: Joachim FREY (joachim.frey@vbi.unibe.ch)

Contribution:

A sensitive detection method based on real-time PCR that allows quantifying *F. tularensis* in tissue samples was developed. Using this method, we identified the spleen and the kidney as the most heavily infected organ in two common squirrel monkeys (*Saimiri sciureus*) from a zoo that died of tularaemia. Spleen and kidney contained up to 400 *F. tularensis* bacteria per simian host cell. In other organs such as the brain, *F. tularensis* was detected at much lower titres. The strain that caused the infection was identified as *F. tularensis* subsp. *holarctica* biovar I. It is susceptible to erythromycin. The high number of *F. tularensis* present in soft organs such as spleen, liver and kidney represents a high risk for persons handling such carcasses and explains the transmission of the disease to a pathologist during post-mortem analysis. Herein, we show that real-time PCR allows a reliable and rapid diagnosis of *F. tularensis* directly from tissue samples of infected animals, which is crucial in order to attempt accurate prophylactic measures, especially in cases where humans or other animals have been exposed to this highly contagious pathogen.

Publications:

Abril C, Nimmervoll H, Pilo P, Brodard I, Korczak B, Markus S, Miserez R, Frey J. (2007) Rapid diagnosis and quantification of *Francisella tularensis* in organs of naturally infected common squirrel monkeys (*Saimiri sciureus*). Vet. Microbiol. in press.

Collaborations:

Dr. Anders Johansson, Dept. Infectious Diseases, Umeå University Hospital, SE-901 85 Umeå, Sweden

15) Name: Cor D. Schoen (cor.schoen@wur.nl)

Contribution:

We report that diagnostics and disease-management strategies require technologies to enable the simultaneous detection and quantification of a wide range of pathogenic microorganisms. Most multiplex, quantitative detection methods available suffer from compromises between the level of multiplexing, throughput and accuracy of quantification. Here, we demonstrate the efficacy of a novel, high-throughput, ligation-based assay for simultaneous quantitative detection of multiple plant pathogens. The ligation probes, designated PRI-lock probes, are long oligonucleotides with target complementary regions at their 5' and 3' ends. Upon perfect target hybridization, the PRI-lock probes are circularized via enzymatic ligation, subsequently serving as template for individual, standardized amplification via unique probe-specific primers. Adaptation to OpenArrays™, which can accommodate up to 3072, 33 nl PCR amplifications, allowed high-throughput real-time quantification. The assay combines the multiplex capabilities and specificity of ligation reactions with high-throughput real-time PCR in the OpenArray™, resulting in a flexible, quantitative multiplex diagnostic system. The performance of the PRI-lock detection system was demonstrated using 13 probes targeting several significant plant pathogens at different taxonomic levels. All probes specifically detected their corresponding targets and provided perfect discrimination against non-target organisms with very similar ligation target sites. The nucleic acid targets could be reliably quantified over 5 orders of magnitude with a dynamic detection range of more than 10⁴. Pathogen quantification was equally robust in single target versus mixed target assays. This novel assay enables very specific, high-throughput, quantitative detection of multiple pathogens over a wide range of target concentrations and should be easily adaptable for versatile diagnostic purposes.

Publications:

Ronald van Doorn, Marianna Szemes, Peter Bonants, George Kowalchuk, Joana Salles, Elen Ortenberg, and Cor D. Schoen (2007) Quantitative multiplex detection of plant pathogens using a novel ligation probe-based system coupled with universal, ultra-high-throughput real-time PCR on OpenArrays™, *BMC Genomics*, **8**:276.

Collaborations:

with Tanja KOSTIC (tanja.kostic@arcs.ac.at) and Levente BODROSSY (levente.bodrossy@arcs.ac.at)

4.2.1 Results of WG2 meetings

Introduction.

The workgroup antigenicity includes 7 laboratories from 6 countries. The WG is coordinated by the Institute of Immunology in Luxembourg. First exchanges and contacts were established during and after the WG meeting in Bratislava. Ongoing research from different partner laboratories was presented as oral communications and provided the basis for new collaborations. The presentations in Bratislava and Antalya covered antigenicity issues of

several pathogens and different approaches to identify pathogen-specific antigens. Some of these antigens were displayed on synthetic carrier molecules or were expressed as recombinant constructs used as diagnostic antigens or used to induce antigen-specific immune-responses in animals. Results of different groups will be presented later in a COST B28 booklet.

1. The Unité des Rickettsies CNRS UMR 6020 - IFR 48 - Faculté de Médecine, Marseille, FR (P. Renesto; patricia.renesto@medecine.univ-mrs.fr)

Contribution

We investigated antigenic properties of Rickettsiae. New transcriptomic and proteomic studies of these intracellular bacteria responsible for epidemic and endemic typhus (*R. conorii* and *R. prowazekii*, respectively), and for spotted fever have been initiated since the complete genome of these pathogens is available. Proteomics strategies were developed to identify rickettsial proteins by accessing facilities including MALDI-TOF, Ion-Trap mass spectrometer and nano-HPLC. This allowed the construction of the first proteomic reference map of both *R. conorii* and *R. prowazekii* and to identify highly antigenic proteins. Putative ligands recognized by endothelial cells could be identified. Another post-genomic application was the analysis of *R. conorii* transcriptome by microarrays. This research aims at further expanding our knowledge about the proteins (actually) expressed by these microorganisms and encoded by virtual annotated genes and which would constitute new diagnostic or therapeutic targets. The group has further characterised of what so far are only putative genes, termed ORFans, of Mimivirus, the largest known virus in particle size and genome complexity (1.2Mb genome) and a possible pathogen responsible for pneumonia.

In 2007 the following results were obtained:

Our strategy to investigate the virulence of the *Rickettsia* genus as human pathogens is now based on post-genomic approaches including both proteomics and transcriptomics. Availability of genome sequences has in turn stimulated the proteomic analysis of these pathogens through two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) combined with high-throughput matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. Using such an approach, we established the first proteome reference maps of both *R. conorii* and *R. prowazekii*. Most recently, we analyzed the proteome of *Rickettsia felis* using two complementary proteomic approaches, namely 2D-PAGE coupled with MALDI-TOF, and SDS-PAGE with nanoLCMS/MS. This strategy allowed identification of 165 proteins and helped to answer some questions raised by the genome sequence of this bacterium.

Recognition of and binding to the host cell is a key step in the pathogenesis of many virulent bacterial strains, and identification of the molecular basis of rickettsial attachment to host cells remains an important objective. This is particularly true when considering the fact that these strictly intracellular bacteria must enter host cells to replicate and survive. In a recently published work aimed to identify rickettsial ligand(s) that bind host cell surface proteins, biotinylated endothelial cells were used to probe a nitrocellulose membrane containing rickettsial extracts separated by 2D-PAGE. Prokaryote candidates recognized by host cells were identified by MALDI-TOF analysis. One protein was identified as the C-terminal extremity of rOmpB called the \square -peptide. The second interacting protein was identified as a protein of unknown function encoded by RP828 in *R. prowazekii* [Renesto *et al.*, 2006]. This protein exhibits a significant sequence similarity to a paralogous ORF (RP827, E-value = 1×10^{-28}) of unknown function. Given the presence of a signal peptide in RP827 (Adr1) and

RP828 (Adr2) and their significant sequence homology with membrane proteins, they are very likely forming a β -barrel structure within the rickettsial outer membrane, a location consistent with its function as putative adhesins. Like the β -peptide, Adr1 and Adr2 are ubiquitously present within the *Rickettsia* genus and might play a critical role in their pathogenicity. To our knowledge, the most recent clinical strain of *R. prowazekii* available around the World was isolated at the Unité des Rickettsies – CNRS UMR 6020 from a blood sample taken from a patient who returned from Algeria in 1999 (strain URRPM22) [Birg *et al.*, 1999. Clin. Microbiol. 37:3722]. To build a list of potential virulence factors of *R. prowazekii*, the annotated genome sequence of this recent clinical strain of *R. prowazekii* was compared with the previously determined genome sequence of the avirulent *R. prowazekii* strain (paper in preparation). Only minor differences were evidenced. Among them, we noticed an insertion in the gene encoding for Adr1. Altogether, the results summarized above are consistent with a key role of Adr1 and Adr2 in the initial steps of rickettsial infection. Accordingly, the goal of our project is now:

(a) To firmly demonstrate the involvement of putative rickettsial ligands into eukaryotic cell invasion, taking in consideration the fact that, as previously mentioned, the genetic transformation of these bacteria is still impossible.

(b) To identify the eukaryotic proteins interacting with such bacterial ligands either by affinity chromatography or by the yeast two-hybrid approach.

This work will require the production of recombinant proteins and of antibodies and will promote collaborations with others laboratories of the WG2.

Transcriptome analysis, such a functional post-genomic application is very useful to understand how pathogens adapt to their environment and has provided some insights into the pathogenic strategies displayed by several micro-organisms during the infectious process. However, DNA microarray-based gene expression profiling for the study of the bacterial transcriptome in course of host cell infection have been hampered by several challenges. In collaboration with Jacques Schrenzel Laboratory (WG1, University of Geneva), we circumvented the difficulty to obtain high yield and quality of RNA from obligate intracellular bacteria, thus allowing the first global transcriptome analysis for rickettsiae. Of note is that, while classified as major bioterrorism agents (CDC), to date, only DNA hybridization experiments were reported for bacteria belonging to *Rickettsia* genus. We believe that this work represents a major achievement which would undoubtedly favour knowledge of these bacteria (and of other intracellular micro-organisms). To assess both the feasibility and the accuracy of our method in monitoring the transcriptional changes of rickettsiae by microarrays, a microarray containing only a limited numbers of targets was constructed. It is now possible to apply the same principles described toward analysis of rickettsial whole genome microarrays. In this respect, the design of the rickettsial probes corresponding to the whole ORFs as well as intergenic spacers has been achieved (A. Huygue and P. François J. Schrenzel Lab) and experiments are in progress.

Publications

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Collaborations.

Jacques Schrenzel (Geneva, Switzerland) for microarray design and analysis of data

3. The Laboratory of Peptide Chemistry, Department of Chemistry, University of Ioannina, GR (M. Sakarellos-Daitsiotis; msakarel@cc.uoi.gr)

Contribution

We developed sequential oligopeptide carriers (SOC_n) for anchoring multiple copies of antigenic/immunogenic peptide epitopes. Carriers formed by the repetitive Lys-Aib-Gly moiety display a predetermined 3D structure (310 helix), while the epitopes anchored by the Lys-NεH₂ groups, preserve their original conformation. The carrier conformation ensures the presentation of antigenic epitopes as potent immunogens. Different SOC constructs were used to induce high titers of specific antibodies recognizing the priming construct and the target antigen. These molecules can also be used for sensitive and specific immunoassays (Artificial Carriers: A Strategy for Constructing Antigenic/Immunogenic Conjugates. Maria Sakarellos-Daitsiotis*, Dimitrios Krikorian, Eugenia Panou-Pomonis and Constantinos Sakarellos *Current Topics in Medicinal Chemistry*, 2006, 6, 1715-1735).

Based on the Bioinformatic Analysis developed by Veljko Veljkovic and co-workers the core sequence of a peptide that belongs to the N-terminal sequence of H5 – VIN1, which represents the prototype antigen for the universal anti-H5N1 vaccine, was coupled in two copies on a synthetic carrier (SOC₄) to use it in immunoassays and vaccine preparations.

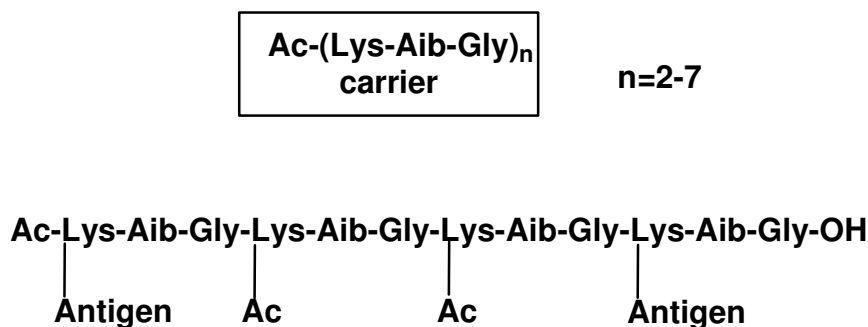


Figure 1. Sequential Oligopeptide Carriers, SOC_n. Antigen: sequence 54-69 of HA1.

In 2007,

Design and synthesis of antimicrobial peptides: Conformational and biological studies. This member continued to developed sequential oligopeptide carriers (SOC_n) for anchoring

multiple copies of antigenic/immunogenic peptide epitopes. Carriers are formed by the repetitive Lys-Aib-Gly moiety and display a predetermined 3D structure (310 helix), while the epitopes anchored by the Lys-N ϵ H₂ groups, preserve their original conformation. The carrier conformation ensures the presentation of antigenic epitopes as potent immunogens. The approach is based on the synthesis and study of helical sequential polypeptides (Arg-X-Gly)_n, where X=Ala, Val, Leu and amphiphilic Aib-containing peptide models of various chain-lengths, Ac-(Aib-Arg-Aib-Leu)_n-NH₂ (n=1- 4). The presence of Aib induces and stabilizes helical structures in combination with the positive charge of the side chain of Arg for interaction with the negatively charged phospholipid membranes. These peptides are further investigated as potential new antimicrobial compounds. The peptides were tested for their antimicrobial activity, their proteolytic stability and hemolytic activity. Conformational studies of the peptides included CD spectroscopy.

Publications

1. Maria Sakarellos-Daitsiotis, Dimitrios Krikorian, Eugenia Panou-Pomonis and Constantinos Sakarellos Artificial Carriers: A Strategy for Constructing Antigenic/Immunogenic Conjugates *Current Topics in Medicinal Chemistry*, 2006, 6, 1715-1735.
2. Djordjevic A, Veljkovic M, Antoni S, Sakarellos-Daitsiotis M, Krikorian D, Zevgiti S, Dietrich U, Veljkovic N, Branch DR. The presence of antibodies recognizing a peptide derived from the second conserved region of HIV-1 gp120 correlates with non-progressive HIV infection. *Curr HIV Res.* 2007, 5:443-8.
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Collaborations:

Based on the bioinformatic algorithms developed by Veljko Veljkovic and co-workers.

4. The Institute of Immunology, Laboratoire National de Santé, LU (C.P. Muller; claude.muller@LNS.ETAT.LU)

Contribution:

We work on conjugate vaccines to protect against viruses and environmental risk compound. The technology could also apply to pathogens of interest of the COST Action B28. One vaccine is based on linear neutralizing epitopes of the measles virus; the other one on derivatives of carcinogen benzo[a]pyrene to explore an immunoprophylactic approach.

1 A peptide-conjugate vaccine. Two B cell epitopes (BCE) of the measles virus (MV) hemagglutinin protein were identified, which can be mimicked by short peptides. Peptides derived from these epitopes induced virus neutralising and protective antibodies even in the presence of pre-existing anti-MV antibodies. The structure of both epitopes was investigated with mabs, substitution peptides libraries and phage display libraries. Since chemical stabilisation of the peptide in the antibody-induced conformation was difficult, and resulted in low-level neutralising antibodies a recombinant approach was used to generate a small permutational library of multiple copies of the epitopes to high-molecular weight polyepitope constructs (<50 kDa). Proper conformation and antigenicity of the polyepitope was confirmed by different methods. Some of the permutational polyepitopes generated high titers of neutralizing antibodies even against all wild-type viruses with mutations in the BCE.

2 Hapten conjugate vaccine. The second example was a hapten conjugate vaccine against benzo[a]pyrene. Monoclonal antibodies were generated by immunisation with a benzo[a]pyrene (B[a]P)-carrier conjugate. The results of this study provide the basis for an immunoprophylactic approach against B[a]P induced immunotoxicity and carcinogenesis.

3 2D-Western detection of viral protein. In order to improve the detection of antigenic proteins on 2D gels and their subsequent identification, a method based on the combination of 2D-DIGE and multiplex western blot was developed. Fluorescently labelled proteins of virus infected cells were separated on 2D gels, blotted on PVDF membranes and viral antigens are detected by monoclonal or polyclonal antibodies and fluorescence-labelled secondary antibodies. The use of 3 different fluorescence labels allows for multiplexing on a same 2D Western blot (ECL-Plex) and for an unambiguous spot overlay prior to the protein identification by MALDI TOF/TOF analysis. Since small 2D-gels with a high density of protein spots can be used for unambiguous spot identification, this approach allows to detect antigenic proteins from microgram amounts of total cell extract. This method provides a very sensitive tool to detect and characterize post-translationally modified species of pathogen- and host cell proteins. Considering the functional importance of such protein modifications as determinants for viral virulence and modulators of mechanisms of the innate immune response, this method is of great interest for the investigation of BSL3/4 pathogens.

4 HPAI Avian influenza virus. During the recent outbreak of avian influenza in Africa the Institute of Immunology established the first laboratory in Nigeria and most of W-Africa with the capacity to diagnose H5N1. Phylogenetic analysis based on full length genome analysis of the viruses isolated on farms in the South-West of the country and in the Northern States represented three lineages and showed evidence for multiple introductory events in Nigeria.

The viral haemagglutinin cleavage-site sequence PQGERRRKKRG was identical to that of the highly pathogenic strains already found in Europe, Russia and Central Asia.

In 2007 we worked mainly on:

1. *Spread and evolution of HPAI H5N1 in poultry, humans and wild-birds in Subsaharan Africa.* During the recent outbreak of avian influenza in Africa the Institute of Immunology established the first laboratory in Nigeria and throughout most of W-Africa with the capacity to diagnose H5N1. During the reporting period the genetic, antigenic and geographic evolution of H5N1 in Africa was further investigated both in poultry and in wild birds. An estimate of the mutation rate of H5N1 in a poultry farm was calculated.

2. *Genetic characteristics of new HBV strains in Subsaharan Africa and SE-Asia: new subtypes, unclassifiable strains and multiple double and triple recombinations.* Sub-Saharan Africa suffers from an excessively high endemicity of hepatitis B virus (HBV), but until recently little was known about the prevalent genotypes. In this study, we investigated the preS1/preS2/S genes of >200 viruses, >150 preCore/Core gene sequences and representative complete genomes collected from 15 locations in Mali, Burkina Faso, Togo, Benin, Nigeria, Cameroon, Democratic Republic of Congo and Central African Republic. Except for Cameroon (18/22 genotype A), >85% of sequences from each location belonged to genotype E with a very low diversity (1.67%) throughout West and Central Africa. In contrast genotype A strains were highly diverse (5.1 %) and separated into three subtypes including two new ones (A4, A5). The low diversity suggests that HBV/E may have a short evolutionary history. It would take only 200 years for the strain diversity of HBV/E viruses to develop from a single introductory event suggesting a short evolutionary history and explaining its conspicuous absence in the New World, despite the forced immigration of slaves from West Africa, until the early nineteenth century. Infection during infancy is mostly associated with chronic carrier status but could hardly account for the explosive spread of virtually identical viruses in Africa. In SE-Asia, detailed phylogenetic analysis of strains from Laos revealed multiple different subtypes of B and C, mixed infections as well as numerous related new strains that are non-classifiable. Both in Africa and Asia a high frequency (>20%) of mixed infections were found and many recombinations between the new non-classifiable. In Nigeria a triple recombination of genotype E/D and A was found.

3. *Incidence of BSL 2 and BSL 3 pathogens in ticks in Luxembourg.* In Europe several tick-borne pathogens classified as BSL 2 or BSL 3 lead to serious infections in humans. Although the interest in incidence data these pathogens increases steadily, currently only incomplete data are available for Europe and none for Luxembourg. This project aims to determine the prevalence of nine tick-borne pathogen genera in Luxembourg. Six of the investigated genera are classified as BSL 2 (*Anaplasma* sp., *Bartonella* sp., *Borrelia burgdorferi* s.l., *Ehrlichia* sp., *Rickettsia* sp., *Babesia* sp.). The human pathogenic species of the other 3 genera are classified as BSL 3 (*Coxiella* sp., *Francisella* sp., TBE virus). Ticks from more than 30 sites throughout Luxembourg have been collected and morphologically identified. The different genera will be analysed by PCR using specific primers. Species identification will then be performed by sequencing. The most commonly reported tick-borne infection is Lyme borreliosis, caused by *Borrelia burgdorferi* sensu lato.

4. *Virus - host cell interactions investigated with gel based differential proteomics, ECL Plex western blot analysis and confocal imaging.* Measles virus (MV) induced immune suppression is only partially understood and accounts for many complications caused by co-infections with other pathogens. We investigated the cellular proteome of a human monocyte/macrophage cell line (THP1) infected by MV vaccine strain to better understand virus-host interactions and molecular mechanisms responsible for differences in virulence among different wild type viruses which need to be further characterized. We used a 2D-gel

based comparative proteomics approach (2D-DIGE) based on the specific and differential fluorescence labeling of mock- and virus-infected protein extracts before co-electrophoresis in a same 2D gel. This method reduces experimental variations and ensures efficient identifications of biological variations in the compared proteomes. To apply this method to limited samples, we have used small 2D gels for DIGE studies. These gels were also used for the preparation of 2D Western blots, using a novel antigene detection system, based on fluorescence labeled secondary antibodies. (ECL Plex technology). The proportion of infected cells was monitored by flow cytometry of viral antigens on the host cells. Beyond 36 hours post-infection cell mortality and protein degradation of viral proteins increased. Differentially expressed protein spots were trypsin-digested and identified by MALDI-TOF mass spectrometry on an Ultraflex I TOF/TOF instrument (Bruker Daltonics). Based on these identifications the localization of cellular MxA and viral nucleoproteins was made by confocal microscopy and completed by quantitative PCR. Our results illustrate that this 2D mini-gel based western blot applies to 1-10microgram protein extracts and that high resolution fluorescence imaging allows to exploit these dense protein patterns to yield information on specific pathogen and host cell proteins, including information on the diversity of post-translational modified forms.

5. *A peptide-conjugate vaccine.* As in 2006 we continued working on conjugate vaccines to protect against viruses and environmental risk compound. The technology applies also to pathogens of interest of the COST Action B28. During the reporting period the laboratory focused on ways to chemically stabilize sequential epitopes. This work is still in progress.

6. *Hapten conjugate vaccine.* As in 2006 we continued working also on these vaccines. We have tested the potential of these vaccines to protect against chemical carcinogenicity in vitro. These results of this study provide the basis for an immunoprophylactic approach against B[a]P induced immunotoxicity and carcinogenesis.

Collaborations

- N. Silman, D. Frangolidis: Support for the planning of the new BSL3 facility of the Institute of Immunology.
- V. Veljkovic and co-workers: bioinformatical analysis of current strains of AIV in Africa.
- Prof. T. Kantardjiev, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria: Investigation of BSL2 and BSL3 pathogens in ticks in Bulgaria.
- J. Eichler (Helmholtz Centre for Infection Research): Joint study of peptides epitopes.
- M. Elschner (Bundesforschungsinstitut für Tiergesundheit, Institut für Bakterielle Infektionen und Zoonosen): Contribution to the strain collection database

Publications

1. CA Bodé, CP Muller, A Madder. Validation of a solid phase-bound steroid scaffold for the synthesis of novel cyclic peptidosteroids. *J Pept Sci* 13, 702-708, 2007.
2. MF Ducatez, CM Olinger, AA Owoade, Z Tarnagda, MC Tahita, A Sow, S De Landtsheer, W Ammerlaan, JB Ouedraogo, ADME Osterhaus, RA Fouchier, CP Muller. Molecular and antigenic evolution and geographical spread of H5N1 highly pathogenic avian influenza viruses in western Africa. *J Gen Virol* 88, 2297-306, 2007.
3. C Bekondi, CM Olinger, N Boua, A Talarmin, CP Muller, A Le Faou, and V Venard. Central African Republic is part of the West-African hepatitis B virus genotype E crescent. *J Clin Virol.* 40, 31-37, 2007.
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- immunoassays and diagnostic impact of mutations in the preS/S gene. *Med Microbiol & Immunol* 196, 247-252, 2007.
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 6. Ren Zhang, Yan Deng, Claude P. Muller, Zhi-Ying Ou, Li Ma, Min Wang^a, Pei-Qiong Li^a, Yun-Shao He. Determination of Hepatitis B Virus Genotype by Flow-through Reverse Dot Blot. *J Clin Virol* 39, 94-100, 2007.
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 8. CP Muller. H5N1. From the front lines: Nigeria. *Nature, Nature News* 440,726-727, 2006.
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 10. F Roman, W Ammerlaan, JM Plesséria, SS Deroo, V Arendt, CP Muller, F Schneider, R Hemmer, and JC Schmit. A new recombinant virus system for the study of HIV-1 entry and inhibition. *J Virol Meth* 131, 99-104, 2006.

5. The Yıldız Technical University, Bioengineering Dept., Istanbul, TK (Sevil Dinçer)

Contribution:

We used phage display technology based on filamentous phages presenting peptides or proteins to produce affinity reagents such as scaffold proteins, antibodies, or inhibitors. Displayed proteins can be used in protein arrays, separation, drug development, epitope mapping and protein-protein interactions. The technology will be applied to the selection of peptides specific to *mycobacterium tuberculosis* and use these peptides as probes in the detection of the related bacteria.

Publications

Türk M, Dinçer S, Pişkin E. Smart and cationic poly(NIPA)/PEI block copolymers as non-viral vectors: in vitro and in vivo transfection studies. *J Tissue Eng Regen Med.* 2007 Sep;1(5):377-388.

6. The Statens Serum Institut, Denmark, (N. Helena Beyer/N. Heegaard)

Contribution:

We produce monoclonal antibodies and polyclonal sera for the development of sensitive detection tests for several neuro- and cytotoxins.

6.1 Botulinum neurotoxin. Several antibody-producing clones against botulinum neurotoxin of serotype A (BoNT/A) have been obtained and the mAb will be available soon. Mice have immunized with BoNT B and BoNT E peptides to generate mAbs against neurotoxins of other serotypes. Polyclonal rabbit antibodies against BoNTs A, B, C, D, E, F have been

produced and are available. An ELISA for detection of BoNT A and BoNT B with L.O. D. of 625 pg is ready.

6.2 Ricin. Rabbit polyclonal antibodies against ricin A- and B-chain have been produced and these antibodies are available. An ELISA for the detection of ricin with a L.O.D. of 1.6 ng is in place. Furthermore, experiments are ongoing to determine whether polyclonal antibodies against ricin A- and B-chain have any protective effect in case of intoxication.

7. The SVB-Dr Victor Babes Hospital for Infectious and Tropical Diseases and the FVB-Dr. Victor Babes Foundation, Bucharest, Romania (Maria Nica, centru@cdt-babes.ro)

Contribution:

We are developing a clinical & epidemiological surveillance program for infectious diseases with bioterrorist impact. 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).

The study aimed the evaluation and connection of some national hospitals for infectious diseases, in order to obtain an organized response in case of unusual situations in order to improve the recognition of critical biological agents.

A randomised selection of 200 cases of acute infectious diseases were presented at FVB or SVB and the patients were classified according to syndromes using the high risk sub-syndromes definition for agents of group A, B and C (CDC classification for critical biological agents). Clinical and epidemiological analysis was based on biostatistical parameter analysis. 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.

The meetings in Bratislava and Antalya were a first important step for the partners in the workgroup Antigenicity to discuss results and complementarities of their research competences in areas important for COST Action B28.

In 2007, the following results were obtained.

We performed Regular work on serology testing for various microbial infections: HIV, HCV, HAV, HBV, HDV, RBV, CMV, HSV, VZV, Measles virus, Rubella virus, *Chlamydia*, *Mycoplasma* *Listeria*, *Borellia*, *Bordetella*, *Brucella*, *Helicobacter pylori*, *Echinococcus*, *Toxoplasma*.

Next to that we have in-house and IVD, classical and real-time PCR technologies for viruses (HBV, HCV, CMV, and HIV-1 viral loads, Polyoma virus, measles virus qualitative PCR) and *M. tuberculosis*. We perform lymphocyte phenotyping and cytokine (Cytometric Bead Array) in biological fluids by flow-cytometry and functional immunology (Quantiferon TB Gold).

Collaborations:

Claude P Muller, Institute of Immunology, National Public Health Laboratory, Luxembourg: Investigation of BSL2 and BSL3 pathogens in ticks in Bulgaria.

Publications:

1. “Genetic profiles of intestinal *Escherichia coli* isolates from Romanian subjects”, C. Usein, M. Nica, A. Palade, N. Popovici, L. Grigore, D. Tatu-Chitoiu, S. Ciontea, M. Damian; 17th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) & 25th International Congress of Chemotherapy (ICC), 2007, Munich, 31.03- 03.04. 2007
2. „Infectii micotice oportuniste la bolnavii imunodeprimati. Rezistenta la substante antifungice”, M. Nica, T. Biolan, E. Mozes, O. Andrei, S. M.Erscoiu, D. Duiculescu, E. Ceausu, P. Calistru; The National Conference of Microbiology, May 24- 26 2007, Mamaia, Romania
3. „Study of the resistant *Streptococcus pneumoniae* strains isolated in Romania between January 2001-June 2007”; Marina Pana, M. Ghita, I. Nistor, R. Papagheorghe, N. Popescu, Maria Nica, S. Botea, Olga Dorobat, V. Ungureanu, I. Apostol, E. Duca, D. Blana, G. Bancescu, D. Leu, M. Andrei, Tibor Osz; 3dr Conference on New Frontiers in Microbiology and Infection-*Streptococcus Pneumoniae*, Switzerland, Oct 7-11 2007
4. “Antibiotic resistance profile in gram-negative bacteria over 25 year period in “Dr. Victor Babes” Hospital, Bucharest”, Nica M., Dorobat O., T. Biolan, E. Mozes, E. Ceausu, P. Calistru, R. Cigoianu; Balcanic Conference of Microbiology, Budva, Muntenegru, Nov 24-26, 2007.
5. “Evaluation of microbiologic risk. Protection methods in laboratory”, Romanian Magazine of Infectious Diseases, No 1-2/2007, ISSN 1454-33-89, pag 17-20; Dr. Maria Nica, Dr. Iuliana Apostol
6. „Food outbreaks with intentional character”, Romanian Magazine of Infectious Diseases, No 1-2/2007; ISSN 1454-33-89; pag 15-17; P. Calistru, I. Apostol, Alma Tudor.

8. Helmholtz Centre for Infection Research, Conformational Protein-Ligand-Interactions, Braunschweig, DE (J. Eichler; Jutta.Eichler@helmholtz-HZI.de)

Contribution:

The overall objective of the research is to develop and implement strategies for the synthetic mimicry of sequentially discontinuous protein binding sites, with the aim to modulate protein function through controlled interference with the underlying molecular interactions. Essentially all biological processes are based on specific binding events, which are initiated by molecular recognition between bio-macromolecules, such as proteins (e.g., receptors, antibodies, enzymes) and their respective ligands (e.g., antigens, hormones, substrates). The structure-based exploration of these interactions is an important element in the understanding of the molecular basis of protein function. The regions of proteins responsible for interacting with the ligand, i.e. the binding sites, are typically located in relatively small, well defined parts of the molecule. The design and generation of molecules, which, due to their specific molecular architecture, can mimic the binding and/or functional sites of proteins, represents a promising strategy for the exploration an understanding of protein structure and function. In addition to this basic significance, such proteinmimetics are also useful tools for a range of biomedical applications, particularly the development of inhibitors of protein-ligand interactions. Protein binding sites are often not located in continuous, consecutive stretches of the amino acid sequence, but rather in parts of the protein that are distant in the amino acid sequence, and brought into spatial proximity by protein folding. The chances of mimicking such sequentially discontinuous protein binding sites through highly flexible, short, linear peptides is rather low, since these peptides are unlikely to adopt conformations required for binding to the respective ligand. Assembled and scaffolded peptides, on the other hand, in which sequentially distant protein fragments are presented in a nonlinear and discontinuous

fashion, can be expected to be better candidates for the mimicry of discontinuous protein binding sites. We have developed solid-phase synthesis strategies for the generation of structurally diverse scaffolded peptides for the synthetic mimicry of discontinuous protein binding sites. The goal of our projects is to mimic the sequentially discontinuous binding sites of a range of biomedically relevant proteins, including interaction domains (WW and EVH1 domains), the cytokine receptor gp130, as well as viral proteins (HIV-1 gp120 and SARS-CoV S1), through such scaffolded and assembled peptides, and to use these molecules as inhibitors of the respective interactions, as well as synthetic immunogens to raise virus-neutralizing antibodies.

Publications 2006-2007 (J. Eichler)

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9. The Centre de Recerca en Sanitat Animal (*Research Centre on Animal Health*) - CreSA, Campus de la UAB, Bellaterra (Barcelona), SP (F. Xavier Abad Morejón de Girón ; xavier.abad@cresa.uab.es)

Contribution:

The institute “Centre de Recerca en Sanitat Animal (CReSA)”, an initiative of Universitat Autònoma de Barcelona (UAB) and Institut de Recerca i Tecnologia Agroalimentàries

(IRTA), is focused on the research, technological development, training and education in the field of animal health.

CRSA has technologically advanced biosafety level 2 and 3 laboratory and *vivarium* facilities. These biosafety units enable scientists to carry out research with pathogenic agents like West Nile virus fever, avian influenza and Newcastle's disease, Tuberculosis, Bluetongue etc. Scientific research in CRSA covers all the disciplines like Immunology, Virology, Molecular and Cell Biology, Bacteriology, Pathology, Parasitology and Entomology. CRSA has facilities to accommodate and perform experimental infection assays with smaller (mice, rats etc) and larger (cows, pigs, sheeps and goats, etc) animals and poultry. The ongoing projects in CRSA that contribute to the COST Action B28 are:

- Experimental infection with avian influenza virus in chicken.
- Environmental persistence and immunopathogenics of the avian influenza virus.
- Epidemiology of swine flu in Spain.
- Hepatitis E virus infection in pigs: diagnosis, epidemiology and pathogenesis.
- Isolation and characterization of bacteriophage of *Salmonella enterica* for their application as agents of biocontrol.
- Risk of the generation of antimicrobial resistance with different medicinal dosages.

Publications:

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3. Olvera A, Cortey M, Segalés J. 2007. Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. *Virology.* 357:175-185.
4. Olvera A, Segalés J, Aragón V. 2007. Update on the diagnosis of *Haemophilus parasuis* infection in pigs and novel genotyping methods. *Vet J.* 174:522-529.

4.2.1 Results of WG3 meetings

Introduction:

The scientific activities of members of workgroup 3 in the year 2006 concerned the application of advanced proteomic and glycomic technologies for the identification of viral or bacterial structures possessing immunostimulatory properties or molecules with unique expression for particular microbial subspecies.

In 2007, Czech and Slovak group have been preparing the training course focused on Proteomics and glycomics. This course will be appointed for ten participants and it will be held in 2008, in Hradec Kralove, Czech Republic.

A booklet will be prepared as has been done for WG1. The booklet focused on basic proteomic approaches and their application for the study of microbial components. It will be done collaboratively with the WG2 members, working on antigenicity, which is a closely related subject. It is currently under preparation and it should be released in 2008

Contributions:

1) Institute of Molecular Pathology, Faculty of Military Health Science, University of Defence, Hradec Kralove, Czech Republic

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 have 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.

In order to identify new immunogenic proteins of *Francisella tularensis* the sub-immunoproteome analysis of membrane-enriched fractions was applied. Furthermore bacteria cultivated under normal and stressful condition were used for membrane collection. By this approach 35 immunoreactive antigens were identified, 15 of them showed to be completely novel immunogens.

The major contribution of this facility in 2007 covers on one side proteomic analysis of stress response of intracellular pathogen *Francisella tularensis*, identification of membrane proteins with immunostimulatory potential and the analysis of intracellular fate of engulfed microbes in macrophages and on the other side development of mass spectrometric and computational procedures for analysis of protein interaction networks.

2) Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia

Coxiella burnetii is the causative agent of Q fever. The bacterium is highly infectious and is classified as a category B biological weapon. A rapid and unambiguous identification of *C. burnetii* is of utmost importance in the localization of naturally occurring Q fever outbreaks or in cases of a deliberate release of the infectious agent. We have developed a Multiple Locus Variable Number Tandem Repeats (VNTR) analysis (MLVA) for an unambiguous identification of *C. burnetii*. The used VNTR markers have revealed many polymorphisms resulting in nine unique MLVA types that cluster into five different clusters. This proves that the MLVA system is highly discriminatory. The developed MLVA method is a promising tool for the characterization of *C. burnetii* isolates and their epidemiological studies.

A new diagnostic preparation for in vitro use has been developed for a rapid and economical serological diagnosis of Q fever using an immunofluorescent (IF) test. Simultaneously, a procedure for its application in the IF test has been developed and standardized. The diagnostic preparation for in vitro use has found a wide application mainly in screening large numbers of sera from human and animals having Q fever.

In 2007, further work has been executed on:

Specific spectral markers were detected in aqueous acetonitrile extracts of the *Coxiella burnetii* (*C.b.*) isolates RSA 493, Priscilla and BUD using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). The measurements revealed characteristic differences in their ion profiles in the given mass range of 3-18 kDa. The highest number of peaks was found for RSA 493 (24) followed by BUD (15) and Priscilla (7). The specific markers observed in the spectra of isolates were matched

with *C.b.* database using Tag-Ident proteomics tool. Eleven potential biomarkers for RSA 493, five for Priscilla and three for BUD have been identified. The system represents a powerful tool for a rapid, sensitive, and differential characterization of *C.b.* isolates and could be a good candidate for phyloproteomic approaches.

The composition and structure of lipid A isolated from the lipopolysaccharide (LPS) of *Piscirickettsia salmonis* were investigated by chemical analyses, gas chromatography/mass spectrometry (GC-MS), and electrospray ionization (ESI) combined with the tandem mass spectrometry (MS/MS). Our study revealed moderate compositional and structural heterogeneity of lipid A with respect to the content of phosphate groups and 4-amino-4-deoxy-L-arabinopyranose (Ara4N) residues and with regard to the degree of acylation. It appeared that at least two molecular species were present in lipid A. The major species represented the hexaacyl lipid A consisting of the β -(1 \rightarrow 6)-linked D-glucosamine (GlcN) disaccharide backbone carrying two phosphate groups. The first one at the glycosidic hydroxyl group of the reducing GlcN I and the second one at the O-4' position of the non-reducing GlcN II. The primary fatty acids consisted of three 3-hydroxytetradecanoic [C14:0(3-OH)] and one 3-hydroxyhexadecanoic [C16:0(3-OH)] acids. The latter was amide-linked to GlcN I and one C14:0(3-OH) was amide-linked to GlcN II. Two secondary fatty acids were represented by C14:0(3-OH) and were equally distributed between the O-2' and O-3' positions. The phosphate group at O-4' carried a non-stoichiometric substituent Ara4N. The minor lipid A species contained exclusively C14:0(3-OH) with an asymmetric distribution (4+2) at GlcN II and GlcN I, respectively. The *P. salmonis* lipid A resembles structurally strongly endotoxic enterobacterial lipid A. This could be one of the reasons for the observed high endotoxicity of *P. salmonis*.

Lipid A isolated from the *Rickettsia typhi* LPS was investigated for its composition and structure using chemical analyses, GC-MS, and ESI combined with MS/MS. The studies revealed that lipid A is composed of two major molecular species that differ one from another in the fatty acid composition. Both species are present in four different tri-, tetra-, penta-, and hexaacylated isoforms having a β -(1 \rightarrow 6)-linked GlcN disaccharide backbone carrying two phosphate groups one at the glycosidic hydroxyl group of the reducing GlcN I and the second one at the O-4' position of the non-reducing GlcN II. In the hexaacylated species, both GlcNs have two amide-linked C16:0(3-OH) and two ester-linked C14:0(3-OH) at O-3 and O-3'. Two secondary fatty acids C16:0 and C18:0 are ester-linked to C14:0(3-OH) and C16:0(3-OH) of GlcN II, respectively. The results showed that both composition and structure of major molecular species of *R. typhi* lipid A resemble those found for the classical forms of enterobacterial lipid A with high endotoxicity.

3) Institute of Nuclear Sciences VINCA Laboratory for Multidisciplinary Research, Belgrade, Serbia

The scientific activity was focused on the development of the EIIP/AQVN bioinformatics platform for structure/function analysis of protein and nucleotide sequences of viral and bacterial pathogens for identification of therapeutic, diagnostic and vaccine targets and selection of drug candidates. This bioinformatics platform was used for (i) identification of conserved determinants of HIV-1 envelope glycoprotein gp120 which are essential for interaction between HIV and the CCR5 receptor, and (ii) identification and valorization of compounds and peptides representing candidate HIV entry inhibitors.

In 2007 the following results were obtained with the work executed in the framework of the COST Action B28:

By applying the informational spectrum method (ISM), a virtual spectroscopy method for the fast analysis of protein-protein interaction and protein structure-function relationships, the protective antigen (PA) of *Bacillus Anthracis* has been investigated. Within the C-terminal region of PA the sequence that is responsible for the interaction between anthrax and its receptor was identified. Similarly, the sequence on the ATR and TEM8 receptors encompassing residues 153 to 185 representing a potential PA binding site was pinpointed. Finally, by applying the ISM approach to the GeneBank protein database followed by solid phase protein-protein assays, the vascular protein EMILIN-1 (Elastin Microfibril Interface Located Protein) was identified as a further major anthrax receptor/binding site. These findings imply that the PA-cell surface receptor interaction is not likely to provide the full explanation for the vascular lesions and prominent hemorrhages that follow *Bacillus Anthracis* infection and spreading and call into play vascular associated proteins such as EMILINs as potential inhibitory proteins.

Collaborations:

- Institute of Virology, Slovakia collaboration with a proteomic group at the Faculty of Military Health Sciences, Hradec Kralove, Czech Republic, a systematic mapping of the *C. burnetii* immunoreactive peptides and proteins recognized by the specific anti-*C. burnetii* human and animal antibodies developed in the course of natural infection will be accomplished.
- There is tight collaboration between Institute of Molecular Pathology, Faculty of Military Health Science, University of Defence in Hradec Kralove and Institute of Virology, Bratislava, Slovakia. This cooperation is based on common scientific project aimed at proteomic analysis of membrane proteins isolated from *Coxiella burnetii* phase I. The preparation of common publication is underway.

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7. Skultety, L., Hernychova, L., TOMAN, R., Hubalek, M., Slaba, K., Zechovska, J., Stofanikova, V., Lenco, J., Stulik, J., Macela, A.: *Coxiella burnetii* whole cell lysate protein identification by mass spectrometry and tandem mass spectrometry. *Ann. NY Acad. Sci.*, 1063 (2005) 115-122.
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4.2.1 Results of WG4 meetings

Contributions:

In Antalya meeting annual results were presented by Pierre Wattiau – Belgium, Prof. Joachim Frey – Switzerland and Stefan Panaiotov from Bulgaria. **Dr. Wattiau** presented a lecture on ‘Single nucleotide polymorphisms as targets for real-time PCR and DNA-array based identification of Bacteria’. He presented his research focused on SNPs as targets for the molecular identification of bacteria at subspecies level. He underlined the fact that often discrimination at the sub-species level rely on SNPs variability. With a few number of properly chosen SNPs, it should be possible to discriminate bacteria belonging to different biovars, serovars and genomovars. He presented description and validation of a group of SNPs for the identification of some biosafety level 3 (BSL3) bacteria. With the methodology presented he is able successfully to discriminate 4 out of the 5 different biovars of *Brucella suis*. He validated these SNPs on a collection of strains by DNA sequencing and Real-Time PCR. Dr. Wattiau working at CODA-CERVA identified additional genetic markers and single nucleotide polymorphisms (SNPs) suitable for the identification and typing of bacteria belonging to the BSL3 containment level. The methodologies used to validate the identified markers and SNPs are: PCR, real-time PCR, Multiplex Ligation-dependent Probe Amplification (MLPA) and low-density microarray screening. The focus is mainly on *Brucella*, *Brukholderia mallei / pseudomallei* and *B. anthracis*. The identification of relevant markers for the development of "thematic arrays" assessing specific subsets of human / animal pathologies is currently investigated.

Another presentation was given by **Prof. Joachim Frey** from the University of Bern on ‘Molecular diversity and antibiotic susceptibility of *Bacillus anthracis* strains causing animal death in Chad: detection of new phylogenetic groups’. In his presentation he described the fruitful collaboration they have with African colleagues. Fifteen *Bacillus anthracis* were isolated from carcasses of cattle in different regions of Chad and were analyzed by use of various markers. Multiple-locus variable-number of tandem repeat analysis (MVLA-VNTR) of eight markers was used to genotype the strains. The analyzed strains formed a novel genetic lineage designated A-beta. Significantly the Chadian anthrax strains were susceptible to 11 tested antibiotics. All tested strains were resistant to ceftiofur, which is a 3rd generation

cephalosporin restricted to animal use. The microarray-based analysis of the DNA revealed the presence of the beta-lactamase genes *bla1* and *bla2* which are endogenous to *B. anthracis*, but not expressed. Besides these two beta-lactamase genes, the strains were shown to be free of all tested antibiotic resistance genes. Prof. Frey concluded that the low 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 suggested that this region contains a unique lineage of *B. anthracis*.

S. Panaiotov presented their experience with the management of an outbreak of Q-fever occurred in Bulgaria in 2004. Bulgarian colleagues reported that the outbreak 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 and was the biggest since 30 years. Serological tests confirmed *Coxiella burnetii* as etiological agent of the pneumonia. Postfactum PCR tests confirmed *C. burnetii* in swab samples. Epidemiological investigations confirmed that the outbreak originated and spread from infected animals. Serum samles from 270 sheep, goats and cattle were tested. 74 of them were positive for *C. burnetii*. The group intends to investigate Rolling Cycle Amplification and Proximity Ligation amplification techniques with the aim to develop specific and sensitive method for *Coxiella burnetii* detection in clinical samples.

Collaborative FP6 projects and bilateral collaborations were established between S. Panaiotov (BG), R. Toman (SK), E. Piskin (TR), and J. Frey (S).

In 2007, WG4 ‘Genomics’ participated in two meetings organized in Plovdiv, Bulgaria (April 2007) and Vienna, Austria (December 2007). Both meetings indicated the main achievements during the year. Research groups reported their scientific and technical outcomes. Several groups reported key results which to some extent changed the European research and management of the high risk BSL3 and BSL4 pathogens. In the recent years, the number of cases of exotic diseases in travellers, immigrants or NGO workers is increasing. During the year an outbreak of brucellosis occurred in Bulgaria. Thus the incidence map of brucellosis in Europe changed. Brucellosis in Bulgaria has been officially eradicated since 1958. Despite this, the closeness of the Mediterranean region, endemic for this zoonotic infection and also the yearly presence of human cases in the neighbour countries created possibility for importing the infection. (**R. Nenova**, Brucellosis – re-emerging infection in Bulgaria, NCIPD, Sofia). Dr. Nenova recommended setting up a program for rapid response at the laboratory level.

Two groups reported fundamental research results on *Francisella tularensis* typing (**Anders Johansson** - Analysis of canonical insertion-deletion markers for safe and rapid DNA-based typing of *Francisella tularensis*, Umea, Sweden and **Ivan Ivanov** - Multiple-Locus Variable-number tandem repeat Analysis (MLVA) for genotyping of *Francisella tularensis* and its application to clinical specimens, NCIPD, Sofia). Both reports underlined the fact that current medical practice does not rely upon subspecies or subpopulation identification, although this information may have predictive value for clinical outcome. Both researchers and groups concluded that rapid and accurate typing methods are needed.

H. Gil and R. Escudero from Madrid, Spain reported ‘Simultaneous detection of *Bacillus anthracis*, *Yersinia pestis* and *Burkholderia* spp. by a multiplex PCR combined with Reverse Line Blot’. It was concluded, that the lack of specificity in the initial symptoms that these microorganisms can cause, requires the design of diagnostic tools for the simultaneous

detection of these pathogens. As an objective they developed a molecular method for the simultaneous and specific detection of *Yersinia pestis*, *Bacillus anthracis*, *Burkholderia mallei*, *B. pseudomallei* and *B. thailandensis*. Selected targets included the capsule gene (*capC*) and the lethal factor (*lef*) for *B. anthracis*, the gene coding the plasminogen activator, (*pla*) for *Y. pestis*, two genes related with the type III secretion system (*orf11* and *BpSCU2*) for *B. pseudomallei* and *B. thailandensis*, respectively, and finally *bimA* for *B. mallei*. The method consisted in a multiplex PCR and a reverse line blot with specific probes for the mentioned targets. The sensitivity was tested with different amounts of genomic DNA (10-10³ genomic equivalents) of the proposed species, and other species of the genus *Burkholderia*, *Bacillus* and *Yersinia*, were used to test the specificity.

Werner Ruppitsch from AGES, Vienna, Austria reported the current research at the Austrian Agency for Health and Food Safety on bioterrorism relevant bacterial pathogens. The aim of their study was to assess the usefulness of partial 16S rRNA gene sequence analysis and the suitability of diverse databases for identifying dangerous bacterial pathogens. In conclusion their study demonstrated that existing information in the databases is not sufficient or reliable for identification of pathogens *Bacillus anthracis*, *Brucella melitensis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Francisella tularensis*, and *Yersinia pestis* by applying 16S DNA sequencing. The obtained sequence data were submitted to three public and two commercial sequence databases for species identification. The most common reason for incorrect identification was the lack of the respective 16S rRNA gene sequences in the database. The group concludes that for discrimination of closely related species sequencing of the entire 16S rRNA gene and additional sequencing of the 23S rRNA gene or sequencing of the 16S-23S rRNA intergenic spacer is essential.

At Vienna meeting new participants from Robert Koch Institute in Berlin and University of East London joined WG4.

Sally Cutler from London, U.K. reported her experience working in Israel on brucellosis typing. She experienced application of VNTR typing strategy on isolates of human and animal origin. She concluded that VNTR technique is powerful approach addressing both the question of identification of brucella species and furthermore the ability of VNTR as an epidemiological typing tool for brucellosis.

Jens Jacob from Robert Koch-Institute in Berlin, Germany experienced molecular differentiation of the genus *Brucella* on the basis of virulence associated genes. He is tempting the hypothesis that the regions around virulence associated genes within *Brucella* species should be selected for construction of microarrays for extensive testing of brucella libraries for genetical differences.

Stefan Panaiotov from Sofia, Bulgaria reported development of AFLP (Amplified Fragment Length Polymorphism) strategy for phylogenetic assignation of species. He reported a whole genome typing technique based on selective amplification of site specific (restriction enzyme digested) DNA fragments in order to create unique fingerprint for a particular genome. AFLP analysis has been shown to be a valuable method for identifying micro(organisms). By establishing an AFLP pattern database one might be able to identify unusual isolates and on the basis of the specific genomic pattern to assign phylogenetically unidentified new species.

Ingmar Janse from RIVM, the Netherlands reported 'Multiplexing the detection of biothreat agents'. He reported his experience in developing molecular detection method for simultaneous identification of several pathogens. Several 4-plex real-time PCR assays detecting three diagnostic targets per organism were examined. At present these assays include *B. anthracis*, *F. tularensis* and *Y. pestis*. He plans to extend the analysis covering species from genera *Brucella*, *Coxiella* and *Burkholderia*. He expanded multiplexing capabilities by developing DNA microarrays as highly parallel diagnostic tools. In order to maintain the speed in the detection procedures, he is currently working with bead-based

suspension arrays. In this microarray format, color-labeled nanobeads coated with probes enable multiplexing of 100 targets which can be rapidly measured by flow-cytometry. The liquid matrix permits rapid reaction kinetics and thus short hybridization times.

An interesting practical work was reported by Pierre Wattiau regarding 'Occurrence and genomic diversity of *Bacillus anthracis* isolation in a wool-processing plant'. He investigated a wool-processing plant for the presence of *B. anthracis* in raw fibers, dust, wastewater and wastewater sludge. Surprisingly 43 *B. anthracis* isolates were identified and typed by VNTR. Identification of different VNTR types suggested multiple sources of contamination origins. No human cases of anthrax were identified, although a serological survey conducted on the employees revealed that some workers had circulating antibodies directed against the anthrax Protective Antigen.

Collaborations:

- **Dr. Panaiotov** coordinated the preparation of a project under the call 'Marie Curie Host Fellowships for the Transfer of Knowledge (ToK) Development Host Scheme' The project was entitled '**Transfer of knowledge for molecular identification, drug susceptibility testing and genotyping of tuberculosis in Bulgaria**'. **J. Frey, E. Piskin and P. Butaye, members of B28, participated in preparintg the section - Biosensors and microarray technologies for drug resistance markers and identification of mycobacteria.**
- **Prof. Erhan Piskin** from Turkey coordinated and in collaboration with S. Panaiotov and Marc Govaerts from VAR-Belgium developed a project entitled „**Design of Novel Miniaturized-Portable-Multichannel Sensors Carrying Aptamers for Detection of Mycobacteria Based on Surface Plasmon Resonance (SPR)**”. The project was under the FP6 scheme: Development of fast tests for diagnosis of poverty related diseases suitable for use in resource-poor settings – STREPs dedicated to SME. Project aim concerned detection of genus Mycobacterium and identification of three most common pathogenic mycobacteria species *M.tuberculosis*, *M. bovis* and *M. avium* subsp. *paratuberculosis* in one test.
- **Stefan Panaiotov and Rudolf Toman** proposed to the Ministry of Education of the Slovak Republic, Division of Science and Technology, Department of Bilateral Cooperation and International Organizations Slovak and to the Bulgarian Science and Technology Co-operation Department for years 2007 – 2009 the project '**Rolling cycle amplification and proximity ligation techniques for sensitive and specific detection of *Coxiella burnetii*, the ethiologicla agent of Q-fever**'. The two years project (2007-2009) was approved by the bilateral Bulgarian-Slovak Science and Technology Commission. Contract will be signed in Feb 2007. Unfortunately for the first two projects experts' evaluation results were very positive, but due to lack of funding or 'very innovative proposed ideas' the projects failed for funding.
- Bilateral exchange programme on Q-fever diagnosis between S. Panaiotov and R. Toman started during this year with the financial support of the Bulgarian and Slovak ministries for Science and Technologies. An exchange visit was performed. More than 70 serum samples were collected by the Bulgarian partner to be processed in the laboratory of Prof. Toman. About 25 strains and other materials will be sent by R. Toman to S. Panaiotov for AFLP typing and comparison of the results previously obtained with VNTR.
- FP7 project 'TM-REST' – 'A new platform for fast molecular detection of MDR and XDR resistant strains of *M. tuberculosis* and of drug resistant malaria' was financially supported by the EU Commission. Partner in the project is S. Panaiotov, NCIPD, Sofia, Bulgaria. Kick-off meeting of the project is planed for 02.2008 in Milano, Italy. The main objective of the proposal is:

- To develop, test and validate a specific diagnostic assay on a lab-on-chip-based new platform (In-checkTM) for the molecular diagnosis and monitoring of tuberculosis and its drug-resistant variants, and for the support and guidance of therapeutic interventions. This tool will allow the identification of MDR and XDR forms by the use of (previously) identified/selected genomic markers and will provide preliminary strain genotyping information thus representing a real improvement over the existing technology. The integrated PCR and Microarray lab-on-chip and the solid-state microarray optical reading tool represents a clear innovation over the conventional readers for its robustness, simplicity of use and low-cost.

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4.2.1 Results of WG5 meetings

The activities of WG 5 have focussed on exchange of strains For facilitating these exchanges, a discussion on a common culture collection has been started. The collection would be decentralised, each laboratory would keep its own collection but information on it would be collected in an excel or access database. It will also include the person to contact and the characteristics of the strains. During the next year this topic will be further worked out and implemented.

Another specific point on which the WG group is working is the extension of the current collections. This is especially true for the more exotic viruses and bacteria, like strains of Viral haemorrhagic fever viruses. Plans are made for the invitation of researchers from areas where the diseases are present and who have a strain collection they want to share. Problems for sharing the collections may be of financial (in general the strains come from developing countries).

Working with live BSL3 and BSL4 agents is not easy. Since the opportunities to work under such conditions within an EU context are rather limited, and since the different countries have different legislations and likewise ways of working in these laboratories, it was judged that a training school, offering an intense training on working under BSL3 and BSL4 agents would be given. The international character of the COST Action B28 will offer the possibilities for the young students to come into contact with the different ways of working in the different

countries and will offer them a comparative basis for improving the safety regulations in their laboratories. The organisation of this will be further discussed in the April meeting in Bulgaria, where it will be decided whether a separate WG meeting would be necessary to further organise things.

During the meetings, main accent of the presentations was on the epidemiology of the BSL3 and BSL4 agents. In depth studies diagnostic limitations of current tests were highlighted. Limitations and possibilities of strain typing were demonstrated by practical studies.

The activities of WG 5 in 2007 have focussed on further collection of data for a common strain culture collection data base (SCDB) and training school. The SCDB contains at the moment about 660 items and is available for downloading from the home page of the Cost B28 action, WG5.

The most important activity of WG5 was the organisation of a training school. Working with live BSL3 and BSL4 agents is not easy. Since the opportunities to work under such conditions within an EU context are rather limited, and since the different countries have different legislations and likewise ways of working in these laboratories, it was judged that a training school, offering an intense training on working under BSL3 and BSL4 agents was given. The international character of the COST Action B28 offered the possibility for the young students to come into contact with the different ways of working in the different countries and will offer them a comparative basis for improving the safety regulations in their laboratories.

A 3-day training school on working under BSL3 conditions was held at Göttingen. 10 students from COST Action B28 participant laboratories from Bulgaria, Serbia, Greece, Czech Republic, Luxembourg, Belgium and The Netherlands were trained in half-day lectures and an afternoon practical. The lecturers all were Cost Action B28 members from Sweden, Belgium, UK and Germany. The response by the participants and the institutes that send them was very good and it is planned to repeat the course in 2008.

1. Name: Dimitrios Frangoulidis

Contribution: design, development and validation of a novel Low-Cost-and-Density (LCD)-Microarray for the detecting of *Coxiella burnetii* and other unusual pathogens. One prototype is finished and the sensitivity was determined to be up to 10 genomic copies/ μ l template for IS1111 and 100 copies for *adaA*. Array modifications according to the design of the internal control are just planned.

Publications:

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Collaborations:

with the group of Rudolf TOMAN (Bratislava, Slovak Republic) for further evaluation with different materials and strains.

2. Name: Anders Sjöstedt

Contribution: Design, validation and application of eukaryotic and microbial diagnostic microarrays. Development of bioinformatic tools for the microarray analysis.

Publications:

1. Henrik Andersson, Blanka Hartmanová, Rhonda KuoLee, Patrik Rydén, Wayne Conlan, Wangxue Chen, Anders Sjöstedt. (2006) Transcriptional profiling of host responses in mouse lungs following aerosol infection with type A *Francisella tularensis*. J. Med. Microbiol. 55:263-271.
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4. Patrik Rydén, Henrik Andersson, Mattias Landfors, Linda Näslund, Blanka Hartmanová, Laila Noppa and Anders Sjöstedt. (2006) Evaluation of microarray data analysis methods using spike-in experiments. BMC Bioinformatics. 7:300-317.

3. Name: Raquel Escudero; Pedro Anda

Contribution: A molecular method for the identification of *Rickettsia* species in clinical and environmental samples (Presented in the meeting of 2005, Bratislava); A molecular method for the discrimination between *Francisella tularensis* subspecies and *Francisella*-like endosymbionts (Presented in the meeting of 2006, Antalya).

Publications:

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7. Coral García-Esteban, Horacio Gil, Manuela Rodríguez-Vargas, Xeider Gerrikagoitia, Jesse Barandika, Raquel Escudero, Isabel Jado, Cristina García-Amil, Marta Barral, Ana

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 20. I Jado, M Boñaós, JA Oteo, JF Barnadika, A Toledo, C Gutiérrez, H Gil, R Escudero, AM Martín-Sánchez, E Santana-Rodríguez, M Rodríguez-Vargas, JL Pérez Arellano, P Anda. Asociación de variantes de *Coxiella burnetii* con diferentes manifestaciones

clínicas en la fase aguda. Estudio clínico y ambiental. XII Reunión de la SEIMC, La Coruña, 9 al 11 de mayo de 2007.

Collaborations:

With Pierre Wattiau Veterinary & Agrochemical Research Center (VAR - CODA - CERVA), Brussels, for the exchange of strains of Francisella.

4. Name: N.J. Silman

Publications:

1. J.E. Burton, O.J. Oshota and N.J. Silman :Differential identification of *Bacillus anthracis* from environmental *Bacillus* species using microarray analysis. *Journal of Applied Microbiology* 101 (2006) 754–763
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3. Roy R. Chaudhuri, Chuan-Peng Ren, Leah Desmond, Gemma A. Vincent, Nigel J. Silman, John K. Brehm, Michael J. Elmore, Michael J. Hudson, Mats Forsman, Karen E. Isherwood, Darina Guryc'ova', Nigel P. Minton, Richard W. Titball, Mark J. Pallen, Richard Vipond. Genome Sequencing Shows that European Isolates of *Francisella tularensis* Subspecies *tularensis* Are Almost Identical to US Laboratory Strain Schu S4. *PLoS ONE* 2(4): e352.
4. Charlton S, Herbert M, McGlashan J, King A, Jones P, West K, Roberts A, Silman N, Marks T, Hudson M, Hallis B. A study of the physiology of *Bacillus anthracis* Sterne during manufacture of the UK acellular anthrax vaccine. *J Appl Microbiol.* 2007 Nov;103(5):1453-60.

5. Name: G. Schmoock, M. Elschner

Contribution:

Development a diagnostic DNA-microarray for the rapid and simultaneous identification of the BSL3 agents *Burkholderia mallei*, *Burkholderia pseudomallei*, *Bacillus anthracis* and *Brucella* spp.

6. Name: M.Weidmann, F. Hufert

In 2007, the group in Göttingen has begun the EU funded project FP6-INCO-VHF-diagnostics, which aims at developing diagnostic tools for viral haemorrhagic fevers. A line assay will carry proteins of RVFV, CCHFV, YFV, DENV, EBOZV, EBOSV and MARV and mobile real time RT-PCR will be developed for the same virus for the Smart Cycler System (www.vhf-diagnostics.eu).

A second project was granted by the German ministry of education and research (BMBF). In this project diagnostic tools will be developed for the detection of European arboviruses causing aseptic meningo-encephalitis. A suspension array for the detection of 7 tick borne viruses and a microarray for the detection of 14 tick borne, mosquito-borne and sandfly-borne viruses are on the agenda (<http://www.virologie.uni-goettingen.de/bmbf/>).

A pre-proposal was drafted in cooperation with Karen Kempself (WG1) Jaques Schrenzel (WG1) and Jean-Luc Gala (WG1). The topic of the pre-proposal covers the improvement of pre-microarray steps and was prompted by the presentation of data concerning the

performance of diagnostic microarrays by Karen Kempself at the Cost Action B28 meeting in Plovdiv. Currently there is no appropriate FP7 call for the submission of this draft. It has been submitted for an initial screen to NIAID in the USA.

Publications:

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3. Weidmann M, Schmidt P, Hufert FT, Krivanec K, and Meyer H (2006) Tick borne encephalitis virus in *Clethrionomys glareolus* in the Czech Republic *Vector-Borne and Zoonotic Diseases* (accepted)
4. Weidmann M, Hufert FT, Sall AA (2007) Viral load among patients infected with Marburgvirus in Angola *J Clin Virol* 39(1): 65-6
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7. Name: P. Pilo

Contributions:

Result about the work to *Francisella tularensis* will appear in the WG1 booklet.

8. Name: CODA-CERVA (Patrick Buatye and Frank Vandenbussche)

Contribution:

CODA has a collection of BSL3 strains (both viruses and bacteria) of animal origin available for further investigation. Some of these have been well characterised. DNA of these strains has been sent to several partners. Some strains have been characterised in collaboration with COST partners.

Publications:

1. Vandenbussche F, Vanbinst T, Verheyden B, Van Dessel W, Demeestere L, Houdart P, Bertels G, Praet N, Berkvens D, Mintiens K, Goris N, De Clercq K. Evaluation of antibody-ELISA and real-time RT-PCR for the diagnosis and profiling of bluetongue virus serotype 8 during the epidemic in Belgium in 2006. *Vet Microbiol*. 2007 Nov 7, Epub ahead of print
2. Goris N, Vandenbussche F, De Clercq K. Potential of antiviral therapy and prophylaxis for controlling RNA viral infections of livestock. *Antiviral Res*. 2007 Nov 5, Epub ahead of print
3. Van Dessel W, Vandenbussche F, Staes M, Goris N, De Clercq K. Assessment of the diagnostic potential of immuno-RCA in 96-well ELISA plates for foot-and-mouth disease virus. *J Virol Methods*. 2008 Jan;147(1):151-6.

4. Toussaint JF, Sailleau C, Mast J, Houdart P, Czaplicki G, Demeestere L, VandenBussche F, van Dessel W, Goris N, Bréard E, Bounaadja L, Etienne T, Zientara S, De Clercq K. Bluetongue in Belgium, 2006. *Emerg Infect Dis.* 2007 Apr;13(4):614-6.
5. Roels S, Wattiau P, Fretin D, Butaye P, Vanopdenbosch E. Isolation of *Morganella morganii* from a domestic rabbit with bronchopneumonia. *Vet Rec.* 2007 Oct 13;161(15):530-1.

Collaborations

J schrenzel, exchange of *B. anthracis* strains for array platform testing

TNO, exchange of *B. anthracis* strains for positive control of molecular tests

5. DISSEMINATION OF RESULTS

5.1 Publications and Reports

Abstract books of the WG meetings are available on the website.

A WG booklet is finished by the WG1 and scheduled to be published during 2008. The demand has been submitted to COST.

The preparation on a second booklet by WG3 is ongoing.

The scientific publications are cited in the WG reports. A total of 46 publications were published by the group partially in 2005 till end 2006. For the period till 2007 the number of publications of the partners related to this COST Action B28 reached 159.

5.2 Conferences and Workshops

A new WG and MC meeting is scheduled for September in Romania. WG1

5.3 Web site

The VAR, Brussels, Belgium has established the website displaying information on all WGs. It is mainly dealing with the aims of the WGs. All partners are listed and WG chairs have administrator rights to update their website. Other documents can be added if wanted. The website is also a communication tool for the meetings (forms, agenda, practical information). It gathers also all abstract booklets.

It contains also information on and links to the participating institutions.

5.4 Scientific and Technical Co-operation

Firm contacts have been established between different partners and collaborative plans have been made. Specific details of collaborations between COST Action B28 partners are listed in the results section of each WG.

A firm collaboration with the FP6 funded IP “moltools” has been established. This group is specifically working on high technological array systems. Actually their applications are foreseen for Eukaryotes. Collaboration for working on Prokaryotes has been scheduled.

There is a connection by several partners with the FP6 funded preparatory action “IMPACT”. FP7 project ‘TM-REST’ – ‘A new platform for fast molecular detection of MDR and XDR resistant strains of M. tuberculosis and of drug resistant malaria’ was financially supported by the EU Commission. Project Coordinator is Daniela Cirillo – Milano Italy and partner in the project is S. Panaiotov, NCIPD, Sofia, Bulgaria. The platform includes development and evaluation of microchip for MDR-TB markers. The three years project starts in 02.2008 – 2011.

5.5 Transfer of results

By means of the website, meetings and other bilateral contacts results are disseminated. It was also agreed that each work package will produce a booklet reviewing the current state of the art of research activities and results within their field. A first booklet has been finished.

5.6 Contacts in the ERA

None so far.

6. ECONOMIC DIMENSION

Total Action Budget Spent from start date: 166,372 EURO distributed as follows:

Budget Overview - Report N°4

Action B28

23/02/2008

Meetings

Meeting Type	Date	Place		Cost	Total
MC	30/05/2005	Brussels (BE)		5518.88	
Joint MC/Working Group	21/11/2005	Bratislava (SK)		35527.7	
Joint MC/Working Group	1/10/2006	Antalya (TR)		27948.5	
Joint MC/Working Group	20/04/2007	Plovdiv (BG)		30430.2	
Joint MC/Working Group	10/12/2007	Vienna (AT)		37634.3	
					137,060

STSM

Beneficiary	Date	From	To	Cost	Total
Ms Anetta Hartlova	1/10/2007	Hradec Kralove (CZ)	CH	2425.00	
Ms Margo Molhoek	10/02/2008	Rijswijk (NL)	BE	800.00	
Ms Katarina Slaba	21/10/2007	Bratislava (SK)	CZ	590.00	
Mr Robert Ihnatko	21/10/2007	Bratislava (SK)	CZ	590.00	
					4,405

Workshops

Title	Date	Place		Cost	Total
3rd Management Committee Meeting and Working Group Meetings	1/10/2006	Antalya (TR)		3000.00	
Joint MC and Working Group Meetings	20/04/2007	Plovdiv (BG)		3000.00	
B28 meeting	10/12/2007	Vienna (AT)		2592.83	
					8,593

General Support Grants

Title	Date			Cost	Total
General	22/03/2006			2000.00	
General	1/09/2007			2000.00	

					4,000
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Schools

Title	Date	Place		Cost	Total
P3 training	22/05/2007	Göttingen (DE)		12313.9	
					12,314

166,372

Total Action Budget Spent from 01/01/2007 until 31/12/2007: 92,376 EURO distributed as follows:

Budget Overview

Action B28

23/02/2008

Meetings

Meeting Type	Date	Place		Cost	Total
Joint MC/WG	20/04/2007	Plovdiv (BG)		30430.2	
Joint MC/WG	10/12/2007	Vienna (AT)		37634.3	
					68,065

STSM

Beneficiary	Date	From	To	Cost	Total
Ms Anetta Hartlova	1/10/2007	Hradec Kralove (CZ)	CH	2425.00	
Ms Margo Molhoek	10/02/2008	Rijswijk (NL)	BE	800.00	
Ms Katarina Slaba	21/10/2007	Bratislava (SK)	CZ	590.00	
Mr Robert Ihnatko	21/10/2007	Bratislava (SK)	CZ	590.00	
					4,405

Workshops

Title	Date	Place		Cost	Total
Joint MC and WG Meetings	20/04/2007	Plovdiv (BG)		3000.00	
B28 meeting	10/12/2007	Vienna (AT)		2592.83	
					5,593

General Support Grants

Title	Date			Cost	Total
General	1/09/2007			2000.00	
					2,000

Schools

Title	Date	Place		Cost	Total
P3 training	22/05/2007	Göttingen (DE)		12313.9	
					12,314

92,376