iPROMEDAI Workgroup meeting Patras 27-28 of May 2015

summarized by Dr. J. Sjollema

In the context of WG3 of the ***ipromedai*** COST-action a group of around 17 scientists discussed about in vitro test methods. Participants were:

- Dr. Madalina Georgiana Albu: University of Bucarest
- Prof. HelenaBujdakova: Comenius University in Bratislava
- Dr. Antigoni Foka, School of Medicine, University of Patras (antigonifoka@gmail.com)
- Prof. Veronique Fontaine: Université Libre Bruxelles (ULB)
- Prof. Todorka Gancheva: University of Chemical Technology and Metallurgy, Sofia
- Dr. Nikolaos Giormezis, School of Medicine, University of Patras (giormenik@yahoo.gr)
- Dr. Maria Katsikogianni: University of Bradford
- Dr. Johanna Kirchoff, University of Jena
- Prof. Evagelia Kouskouni: National Kapodistrian University of Athens
- Dr. Filipe Jose Menezes Mergulhao: University of Porto
- Prof. Yannis F. Missirlis: University of Patras
- Dr. Madeleine Ramstedt: Umeå University
- Prof. Erik Reimhult: University of Vienna
- Dr. Qun Ren: EMPA, Switzerland
- Dr. Martijn Riool: University of Amsterdam
- Dr. Jelmer Sjollema: University of Groningen
- Prof. Iris Spiliopoulou, School of Medicine, University of Patras (spiliopl@upatras.gr)

The main objective of ***ipromedai*** is to reduce the risks of device associated infections by a comprehensive, highly transdisciplinary approach, addressing clinical needs and combining novel concepts of surface modifications and improved procedures of testing for antimicrobial efficacy *in vitro* and *in vivo*. Specifically a specific objective was formulated:

› Documentation of comprehensive sets of 2 standard and novel test methods that allow for testing efficacy *in vitro* and *in vivo*

In order to address the improvement of *in vitro* testing a workshop was organized in Patras (Greece) where participants of the Workgroup 3 (Mechanistic Studies, *in vitro* Testing, Sensing and Modelling) discussed present standards, identified short comings and defined uniform conditions for evaluation of novel ideas with respect to *in vitro* testing.

To that end the meeting had the following objective:

To design a draft plan, to be discussed in the ipromedai meeting in Davos, for setting up

- a set of standard bacterial species and strains,
- standard reference materials (best practices, both polymer and metal based) and
- a set of evaluation methods

The first two points were put on the agenda to create controllable conditions for a round robin experiment among a number of participating laboratories to evaluate the suggested novel aspects of *in vitro* testing. Furthermore, suggestions for method changes or novel methods will be submitted to a standard organization for potential standardisation.

In vitro evaluation of antimicrobial testing is challenging because

- the variation of materials to be evaluated is high: including, metals like Titanium and stainless steel, ceramics and technical polymers.
- The variation in applications is diverse: ***ipromedai*** members are working in the area of orthopedics, trauma, catheter technology (both urinary and intravenous), trauma, cardiovascular, dental applications and wound infections.
- Diverse microorganisms are involved, including Gram+ and Gram- species as well as fungi.
- Diverse antimicrobial mechanisms should be evaluated, the three main ones involving contact killing, non-adhesive and releasing materials or material coatings.

In order to limit the above mentioned degrees of freedom, a selection was suggested by the Patras group to focus on in their effort to define optimal evaluation methods:

1: Main applications for which the method should be applicable are

› Catheters (both the intra- and extraluminal part)

› Hip Arthroplasties.

Both applications were mentioned most frequently in a survey among all ***ipromedai*** participants.
In order to define what aspects of both applications are relevant for the setup of in vitro tests, the clinical conditions around the interface of these devices were discussed.
For urinary catheters the following parameters are important:

› Intra- and extraluminal flow (medium to high shear forces).

› Protein adsorption (extra luminal)

› Exposure to immune cells in the entrance part (extraluminal of IVC's)

For hip-arthroplasties the following parameters were found to be relevant for testing:

› Non-stagnant conditions (low shear forces) for body fluids around the implant

› Unknown volume for containment of released anti-microbials

› Protein adsorption

› Exposure to both immune cells and bone and/or tissue cells .

With the selection of these applications the materials to which the test should be applicable are Titanium, stainless steel and polymers, like Ultra High Molecular Weight Polyethylene, Silicone rubber, Poly-urethane and other polymers used in catheters and orthopedic arthroplasties.

2: Main microorganisms selected

Both devices are prone to bacterial contamination and device associated infections. The main bacterial species which were selected by the Patras workgroup are all biofilm formers and usually the main pathogens associated with either of the two applications:

- *Staphylococcus aureus* (orthopedic devices)
- *Staphylococcus epidermidis* (both catheters and orthopedic devices)
- *Escherichia coli* (urinary catheters)
- *Pseudomonas aeruginosa* (both applications)

In order to be able to create uniform conditions for testing in vitro methods, it is advised by the group to select and assess specific strains from commercial collections. Specifically European collections, partly also providing ATCC strains, are recommended. These are e.g.:

- CBS-KNAW Fungal Biodiversity Centre
- CABI
- NCIMB

Although it is recognized that the relevance of antimicrobial evaluation methods should mimic the in vivo situation as well as possible, it was decided not to include immune or tissue cells in a basic test to be selected or developed. This action is particularly addressing a screening procedure where a number of variations is pre-selected for in vivo testing. Moreover introducing immune and tissue cells would highly complicate the evaluation. For the same reason the Patras group will not demand co-cultures of bacteria to be introduced in the test, although it is clear that co-cultures of bacteria may change the efficacy of the antimicrobial material.
It is however recommended that evaluation methods should be designed in a way that they allow extension to a far more complex situation with bacterial co-cultures and/or immune or tissue cells.

3: The main antimicrobial mechanisms the test-methods should be tailored to are:

- Mechanism based on antimicrobial releasing platforms. Antimicrobial releasing materials do not only kill bacteria at the surface (potentially in a biofilm) but also kill on a distance, enabling the eradication bacteria that are migrated to the tissue some tens to hundreds of micrometers away from the surface. One of the drawbacks of releasing systems is the relatively short time the surface is active since release systems get depleted over time.
- Mechanisms based on surfaces with immobilized antimicrobial moieties do not have these drawbacks and potentially keep their activity for a long period of time. Vice versa their drawback is that they do not kill bacteria in the tissue, a bit away from the interface of the implant. Another aspect of antimicrobial surfaces is that dead bacteria at the surface may accommodate other viable bacteria to safely grow out to biofilms.

› Mechanisms that rely on non-adhesiveness towards bacteria. These surfaces may keep their performance for long time, but their functionality is often disputed since protein adsorption, always taking place in case of implants, may disrupt the non-adhesive properties.

In order to take into account the above mentioned constraints regarding micro-organisms, mechanisms, materials and applications, the Patras group formulated a number of requirements the methods should meet.

1. The method should be robust, which means that it should be non-complex, fault tolerant and render reproducible results when executed in different labs.
2. The method should be easy to operate and need no expensive equipment
3. The method should allow and control shear forces to be applied during the test on bacteria attached to the sample. Shear forces need to be quantifiable and in a range that comply with shear forces expected both in catheters (high shear) and hip implants (low but non-zero shear).
4. The method should be extendable to allow cells and bacterial co-cultures in order to better mimic the in vivo situation.
5. The test method should be quantitative, which means that the reduction of viable bacteria from a starting inoculum can be inferred from the test results in terms of decades of reduction. Reduction should be related to control samples (identical substrates without the added functionality) which are treated identical as the experimental samples.
6. The method should allow proving efficacy of at least four decades. The Patras group firmly states that lower reductions are clinically less relevant, mainly because any remaining single bacterium left can cause new infections. This requirement is not valid for non-adhering surfaces. Here no limit is mentioned, but 3 decades should be sufficient.
7. The method should allow both the analysis of the surface (how many viable bacteria are present on the surface, how many are dead (if not released)) and the analysis of the suspension or supernatant which was in contact to the surface.
8. The method should allow evaluation of all three antimicrobial mechanisms.

None of the presently available tests (partly standards) do fulfill all of the above mentioned requirements (see table below).

Requirements	*Robustness*	*Easy*	*Shear forces controlled*	*Extendable*	*Quantitative*	*Allow >=4 decades*	*Surface analysis*	*Mechanisms*[1]
Agar tests	Yes	Yes	No	No	No	N.A.	No	REL
Suspension test (ASTM 2149)	Yes	Yes	No	No	Yes	Yes	No	REL
JIS test (JIS Z 2801)	Yes	Yes	No	No	Yes	Yes	No	REL CMB
Petrifilm[2]	Yes	Yes	No	No	Yes	No	No	REL CMB
Suspension adsorption[3]	Yes	Yes	No	No	Yes	Yes	No	CMB NAD
Flow chamber[4]	Yes	No	Yes	Yes	Yes	Yes	Yes	CMB NAD
FACS [5]	Yes	No	No	No	Yes	Yes	Yes	REL CMB NAD

Two new methods were discussed in which most of the above mentioned requirements were met, one based on flow chambers, the other in rotary shakers. Both enable the control of shear, are robust and do not need expensive equipment.
The Patras group advises that these new methods should be further specified and evaluated in a round-robin experiment around the participants of the ***ipromedai*** consortium.

1 REL: Releasing systems
CMB: Contact Microbicidal
NAD: Non-adhering
2 J. Sjollema *et al.*, Journal of Controlled Release, 188 (2014) 61–66.
3 A. Hequet *et al.*, Colloids and Surfaces B-Biointerfaces 84 (2011) 301-309.
4 D.P. Bakker *et al*. Applied and Environmental Microbiology 69 (2003) 6280–6287.
5 X. Xie *et al*. Biomaterials 32 (2011) 4347-4357.

In order to perform the evaluations the next steps are anticipated and will be discussed in the COST-action Davos meeting end of June:

- Full description of the methods.
- Purchasing single batches for three types of antimicrobial surfaces from companies or institutes including all three types of antimicrobial mechanisms, which serve as standards for the evaluation. Second option is to distribute protocols for participants to produce their own samples.
- Purchase of four standard species/strains from known microbial collections.
- Execution of the evaluation in diverse labs on diverse locations.
- Evaluation of results.
- When either of the tests appear to be appropriate and fulfill most of the requirements submission to European Standard Organization is considered.