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Cover Letter

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To the Editor
of Microbes and Infection

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Lausanne, 29 January 2014

Dear Editor,

Please find attached a manuscript entitled " **ESCMID postgraduate technical workshop on intracellular bacteria: from biology to clinic**", that we submit as an article in Microbes and Infection.

In this meeting report, we present the main events that took place during this meeting and describes main scientific presentations.

This manuscript is not submitted or accepted for publication elsewhere. The current version of the manuscript has been seen and accepted by both authors. We hope that this original work will fall within the scope of your Journal.

Sincerely yours,

Gilbert Greub

canton de vaud

ESCMID postgraduate technical workshop on intracellular bacteria: from biology to clinic

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Abstract

Infection by intracellular bacteria can lead to several diseases in both veterinary and human medicine. Unfortunately, the biology of these intracellular bacteria is highly complex due to their interactions with their host cells. Thus, it is very important to develop several tools in order to better understand the complex intracellular life of these pathogens, so allowing to improve the diagnosis options and the treatments of infectious diseases that they are causing. The workshop organised in Villars-sur-Ollon (Switzerland) by the ESCMID Study group on intracellular bacteria was a good opportunity to enhance our knowledge on these fastidious pathogens. During 5 days, 15 speakers gave 41 talks, covering all fields, from biology to clinic of different intracellular bacteria such as *Bartonella*, *Chlamydia, Coxiella, Ehrlichia, Listeria, Parachlamydia, Risckettsia,* and *Waddlia*. The format of this postgraduate course, which took place in the Swiss mountains, allowed interactive sessions and living discussions between the participants coming from all around the world. One of the major strength was to gather epidemiologists, clinical microbiologists, infectious diseases specialists, entomologists, veterinarians as well as bioinformaticians, biochemists and biologists to deliver a unique "one-health science" on intracellular bacteria. Here, we summarize the main take-home messages delivered during this meeting.

Main text

1) Introduction

Obligate intracellular bacteria, such as *Chlamydia*, *Rickettsia* and *Coxiella*, have to infect their eukaryotic host in order to survive. Some of them are able to infect a wide range of hosts and some may even cause an infectious disease. These infections can be asymptomatic but in most cases, they result in significant deleterious effects for the host, often associated with significant mortality and morbidity. Due to the difficulty to detect obligate intracellular bacteria, they have been often described for the first time during outbreaks. Thus, some intracellular bacteria are likely yet unknown whereas others are still only considered as emerging pathogens since it is difficult to confirm their pathogenic role. Moreover, despite significant pathogenicity of several intracellular bacteria, they are still poorly studied. Indeed, due to the historical lack of genetic tools, and due to the need of cell culture, it is very difficult to study their strict intracellular lifestyle and to precise the virulence factors involved in their pathogenesis. Fortunately, several people are working in this field and try to better understand the biology of intracellular bacteria and the pathogenic mechanisms that are at play.

The ESCMID study group for intracellular bacteria (ESCAR) is currently composed of approximately 300 members, including physicians, microbiologists, veterinarians, and other specialists, interested in the biology, epidemiology, and pathogenicity of *Coxiella, Ehrlichia, Anaplasma, Rickettsia, Bartonella, Chlamydia* and *Chlamydia*-related bacteria. Since the aim of ESCAR is to encourage basic and applied research in the field of intracellular bacteria and related diseases, ESCAR regularly organise postgraduate courses.

Thus, an ESCMID postgraduate workshop was organised by Pr. Gilbert GREUB in Villars-sur-Ollon, in Switzerland, from 26 to 30 August 2013, and it was entitled "Intracellular Bacteria: From Biology to Clinic". This workshop gathered 55 participants and included interactive sessions, fostering living discussions and a high level of interaction and cooperation about our shared fascinating interest, intracellular bacteria. During this workshop, the sessions alternated between i) biology, with talks about the pathogenesis, molecular biology, genomics and cell biology of intracellular bacteria, and ii) medicine, with talks about clinical presentation, diagnostic approaches, epidemiology and treatment of infections caused by obligate intracellular bacteria.

2) Participants and venue

The workshop on intracellular bacteria, with 55 participants coming from 21 countries, was organised in Villars-sur-Ollon, in the beautiful scenery offered by the Swiss Alps. The parity was appropriate during this postgraduate course, with 30 men and 25 women, and with people coming from all around the world. Majority of the participants were from European countries, but some people made a long trip, such as 2 investigators from Korea, 3 from Australia, 1 from India, 1 from Sri Lanka, 2 from Tunisia and 1 from USA. The atmosphere was very relaxed and friendly between all participants, allowing fruitful discussions and interactions, particularly during the lunch and diner breaks around delicious specialities like the famous Swiss Raclette (Fig.1). This week of scientific presentations on intracellular bacteria was brighten up by an afternoon of hiking in the pastures, allowing also to know each other better (Fig.1). For the most motivated people, a jogging session was organised all morning and it was also very nice to discuss and to have the opportunity to create some collaborations in parallel to non-scientific activities (Fig.1). The last day, it was time to come down again to the lemanic area, since this day was dedicated to the practical session organised in Lausanne at the University Hospital and at the Institute of Microbiology of the University. The practicals represented a good opportunity for all participants, distributed in small groups of 5 persons, to discover some techniques used to study intracellular bacteria, and/or to improve their practical skills (see below).

3) Epidemiology

The workshop was opened by the chairman of ESCAR, Pr. Amel Letaief, and by the organizer, Pr. Gibert Greub. During the first afternoon, talks focused on the epidemiology of infections caused by three different obligate intracellular bacteria: *Rickettsia, Coxiella* and *Chlamydia.*

P.E. Fournier (Marseille, France) started this session with an overview of *Rickettsia* present around the world. *Rickettsia* are intracellular bacteria widely distributed and vectorized by arthropods such as ticks, fleas and body lice. These bacteria are human pathogens, which may cause either a spotted fever rickettsial infection or the typhus. As underlined by P.E. Fournier, the dogma associating a given rickettsiosis to a given arthropod vector is false, since exceptions may commonly occur and geographic repartition of rickettsiosis has been recently redefined [1]. Arthropods are key vectors for *Rickettsia* but could also be vectors of others intracellular bacteria. This hypothesis was formulated by A. Croxatto (Lausanne, Switzerland) that reported the results of its project investigating the transmission of *Chlamydia* and *Chlamydia*-related bacteria by arthropods. Thanks to a new pan-*Chlamydiales* PCR, Croxatto et *al*. showed the high

prevalence (30 to 45 %) of *Chlamydiles* present in ticks collected in Switzerland and in Algeria (Croxatto *et al*. in press). Thus, arthropods and more specifically the ticks, already known as vector for *Rickettsiales*, seem to also act as a reservoir and possibly as a vector for some *Chlamydia*-related bacteria. This is important since human and animals are commonly exposed to ticks. Small ruminants are particularly susceptible to chlamydial and rickettsial infections [2]. D. Longbottom (Edinburgh, UK) focused his talk on five strict intracellular bacteria: (i) *Ehrlichia ruminatum* responsible for Heartwater, (ii) *Anaplasma ovis* and (iii) *Anaplasma phagocytophilum* responsible for Anaplasmosis and Tick-borne fever respectively, (iv) *Coxiella burnetii* responsible for Q fever, and finally (v) *Chlamydia abortus* responsible for ovine chlamydiosis. These five ovine infectious diseases were described in details including clinical pictures, and approaches used for their diagnosis and treatment options in the veterinarian field. The zoonotic risk was also addressed, being thus an excellent introduction for the second part of the afternoon sessions dedicated to three *Coxiella* outbreaks.

The prevalence of the Q fever is very low in Switzerland but one of the largest *Coxiella* outbreaks ever described worldwide occurred in 1983 in the "Val de Bagnes", a few dozen of kilometres away from the meeting venue. Up to 415 persons were infected, as underlined by O. Peter (Sion, Switzerland). The source of the outbreak was sheep flocks coming from alpine pastures [3]. More recently, another small outbreak was reported in Lavaux (Switzerland), and G. Greub (Lausanne, Switzerland), who presented this topic, stressed the importance of the fast implementation of different public health measures in order to impact favourably the outcome of outbreaks. Indeed, rapid implementation of public health measures may explain why the outbreak remained limited to only 14 human cases (Bellini *et al*. New microbes and new infections, in press). Unfortunately, outbreaks do not always have such a favourable issue and may last for years. This is the case of the huge Q fever epidemic that started in Netherlands in 2007. As highlighted by C. Bleekers-Rovers (Nijmegen, Netherlands), due to a delay to implement measures and to environmental conditions favourable to bacterial dissemination, the epidemic expanded and more than 4000 cases have been identified to date [4]. The epidemic is now under control but clinicians currently face a subsequent huge outbreak of patients suffering from chronic Q fever. This long lasting Q fever epidemic has been a major opportunity to obtain precise information concerning the control of Q fever outbreaks, the diagnostic, the treatment of the disease and of course, the transmission of *Coxiella*, which may hopefully help public health actors and infectious diseases specialists to avoid another such large epidemic in the future.

4) Clinical presentations, diagnostic approaches and treatments

During all morning sessions, speakers detailed the clinical presentation of infections due to different intracellular bacteria and provided state of the art information on diagnosis approaches and treatment options of these infections. Thanks to the huge Dutch Q fever epidemic, a lot of information about *Coxiella* infections has been gathered [4]. As reported by C. Bleekers-Rovers (Nijmegen, Netherlands), the infection is symptomatic in only about 40% of infected people, that present generally with an acute Q fever. This infection is generally diagnosed by using PCR and/or serology, as underlined by O. Péter (Sion, Switzerland) and is resolved spontaneously within 2 or 3 weeks. Despite that, 1-5% of the patients develop a chronic Q fever, so it is necessary to treat acute Q fever for the patients at risk, with doxycycline 200 mg daily for 21 days. In case of chronic Q fever, the challenge is to early diagnose the infection. Indeed after an asymptomatic phase, severe complications occur with endocarditis as the most common manifestation, leading to high morbidity and mortality varying from 13% with a good treatment to 60% in absence of treatment. The recommended treatment is a long-term association of doxycycline and hydroxychloroquine, and the resection of the infected tissues. Two others intracellular bacteria play also a major role in the development of infectious endocarditis, *Bartonella* sp., and *T. whipplei*. Indeed, as explained by G. Greub (Lausanne, Switzerland), 14 species of *Bartonella* may infect humans, being responsible for several diseases such as Oroya fever (*B. bacilliformis*), cat scratch disease (*B. henselae*), trench fever (*B. quitana*) [5]. This infection is difficult to diagnose, so most of time it is better to use PCR, serology and/or histology. For uncomplicated cases there is no need of antibiotic, but for patients at risk or for specific clinical presentations, personalised antibiotic treatment might be used. *T. whipplei* are also responsible for endocarditis in case of localized chronic infection, but in the majority (80%) of the cases, the clinical manifestations are non specific, different organs can be targeted and when the gut is involved with associated weight loss, malabsorption syndrome and arthritis, we name this syndrome "classical Whipple's disease". (F. Fenollar, Marseille, France). The diagnosis is mainly based on PCR, and the treatment is empiric but long-term (for lifetime) association of doxycycline and hydroxychloroquine seems to be the ideal treatment.

The diagnosis of blood culture negative endocarditis caused by *T. whipplei* and intracellular bacteria such as *Bartonella* and *Coxiella* is often difficult. Indeed, as reported by P-E Fournier (Marseille, France) (Fig. 2) blood culture being negative, it is necessary to use other diagnostic approaches such as PCR on blood and valves, serology and immunohistochemistry (or auto-immunohistochemistry) on valves [6] [7]. Another interesting clinical characteristic of intracellular bacteria is the ability to induce adverse pregnancy outcomes [8]. D. Baud

(Lausanne, Switzerland) (Fig. 2) summarized the role of some intracellular bacteria in humans' and/or animals' miscarriage, stillbirths, and preterm labour. *Listeria monocytogenes* is one of these bacteria. As described by M. Lecuit (Paris, France), this bacterium is very well known as an entheropathogen but it can also be responsible for septicemia, central nervous system infections, and maternal-foetal infections [9]. The diagnosis can be done by culture (blood, cerebro-spinal fluid, placenta,…), PCR, serology, and immunohistochemistry. The recommended treatment is amoxicillin combined to gentamicin for severe infections. Among the other intracellular bacteria implicated in adverse pregnancy outcomes, the importance of *Chlamydia* and *Chlamydia*–related bacteria is increasingly recognised. D. Baud (Lausanne, Switzerland) exposed the characteristics of *Chlamydia trachomatis* infections that include trachoma and urogenital infections. These infections may be diagnosed by PCR or serology, and are best treated with doxycycline or azithromycin. D. Baud presented also some data about *Waddlia chondrophila*, a *Chlamydia*-related bacterium considered as an abortigenic agent in bovine, and associated with human adverse pregnancy outcomes [10] [11].

5) Cell and molecular biology

Several intracellular bacteria are human and animal pathogens. In order to understand mechanisms involved in their pathogenicity, it is essential to study the cellular and molecular biology of these pathogens. To enter, survive and grow within their host cells, intracellular bacteria need to evade the defence mechanisms of these host cells and to generate a replicative niche. As reported by A. Croxatto (Lausanne, Switzerland), secretion systems are one of the tools used by intracellular bacteria to interact with the host cell. Nowadays, seven secretion systems have been identified but Croxatto's talk was focusing on the Type Three Secretion System of the *Chlamydiales* and on the difficulty to identify its effectors. However, it is essential to identify these secreted effectors. Indeed, as explained by J. S. Dumler (Baltimore, USA), the pathogenesis of *Anaplasmataceae* infections is better understood since the identification of type II and type IV secretion systems effectors, which (i) interfere with host membrane traffic, (ii) interact with MAP kinase signalling pathway, and (iii) bind DNA in the nucleus leading to reduced the transcription of important genes [12]. *Chlamydia pneumoniae* are other intracellular bacteria using type III secretion system to modulate and interact with host cell signalling pathways. Within their inclusions, these bacteria are able to evade host defence mechanisms and to survive in a persistent stage resulting in a chronic infection. M. Puolakainen (Helsinki, Finland) (Fig. 2) described the particular way of life of these bacteria and the large panel of pathologies associated with chronic chlamydial infections. Always associated to *Chlamydia* pathogenesis, an important group of proteins specific to *Chlamydia* was described

by D. Longbottom (Edinburgh, United Kingdom). The polymorphic membrane proteins (Pmps), are highly immunogenic and play an important role in bacterial virulence by acting as autotransporter proteins of the type V secretion system [13] [14]. Catalases are another key virulence factor for intracellular bacteria enabling their survival within phagocytic cells, but no catalase have been described so far in classical *Chlamydia*. B. Rusconi (Lausanne, Switzerland) showed the presence of genes encoding for catalases in *Chlamydia*-related bacteria and based on a phylogenetic analysis, she highlighted the important role of these catalases and their evolutionary history in the chlamydial order [15].

Virulence is a key feature of intracellular bacteria, and the panel of strategies deployed by these bacteria is huge. The *Bartonella* genus is composed of 14 pathogenic species, able to infect and multiply within endothelial cells and erythrocytes [16]. Then, as reported by P-E. Fournier(Marseille, France), these bacteria are able to promote angiogenesis allowing dissemination in the host organism, and are vectorized within erythrocytes, by insect vectors such as blood-sucking arthropods [16]. *Rickettsiae* are also transmitted by these arthropods, and then disseminated throughout the body via the bloodstream. These strict intracellular bacteria are able to infect a large panel of cells, but the main targets are endothelial cells. G. Greub (Lausanne, Switzerland) described their capability (i) to corrupt the host cells after escaping to the phagocytosis vacuole, (ii) to induce secretion of proinflammatory cytokines, and (iii) to inhibit apoptosis. Some intracellular bacteria are involved in several serious human pathogenesis such as *Listeria*. M. Lecuit (Paris, France) reported the dual roles of ActA protein involved in the virulence and persistence of Listeria during intestinal infections. Moreover, he detailed the strategy used by *Listeria*, with the interaction between internalin and E-cadherin, to target and cross both intestinal and placental barriers [17]. Finally, pathogenesis induced by intracellular bacteria are sometimes very insidious. As explained by F. Fenollar (Marseille, France), it is the case for *Tropheryma whipplei* pathogenesis. Virtually nothing is known about these bacteria that seems to be opportunistic and might cause chronic infections among genetically predisposed patients.

6) Genomics

The Wednesday afternoon wad dedicated to talks about genomics of intracellular bacteria. Twenty years ago, it was the beginning of genome sequencing, and this tool is now used almost in routine. Nowadays, we are in a new genomic era focusing the advent of genomics of medical importance [18]. We have access to an ever increasing number of genomes and this provides significant information about intracellular bacteria. Genomics provides an insight in bacterial evolution and bacterial metabolism. Moreover, genomics is a

very useful tool for research of new drugs or new drug targets, and provides information on the presence of a variety of virulence factors, including secretion systems, autotransporters, adhesins, and catalases. These virulence factors may be identified by various functional genomics approaches, as outlined by Marie de Barsy [19]. Lessons gathered from genomics of Listeria, Riskettsia, Chlamydia, and Chlamydia-related bacteria have been presented during this session.

7) Practicals

The last day of the workshop was dedicated to 9 different practicals (Fig. 3). They were organised at the Institute of Microbiology (IMUL) of the University Hospital Center (CHUV) and at the School of Medicine of the University of Lausanne.

The main techniques for bacterial identification, isolation, staining and observation have been taught with a special focus on specific phenotypes and tools applied to intracellular bacteria, such as *Legionella* and *Listeria*. Participants had the opportunity to have some explanations about bacterial identification by MALDI-TOF

Amoebal co-culture and amoebal enrichment have also been presented to the participants, to get them familiarized with approaches used to isolate amoeba-resisting microorganisms (ARM) and to recover free-living amoebae from clinical and environmental samples [20] [21]. To show how to obtain cells from different organisms, allowing further functional assays, isolation of mouse Bone Marrow-Derived Macrophages (BMDM) was shown. Ticks dissection was also explained in details. The importance of techniques allowing visualisation of infected cells or bacteria alone was highlighted by a detailed description of the immunofluorescence technique, as well as Gram and Diff-Quick stainings. Finally, bioinformatic tools useful for genome assembly and annotation were presented, emphasizing the importance of an interdisciplinary approach for biological data analysis.

8) Conclusions

This Workshop, organised by Pr. Gilbert Greub on behalf of ESCAR, took place in the heart of the Swiss mountains, in a convivial environment, where biologists and clinicians had the possibility to meet, exchange ideas and learn about epidemiology, genomics, diagnosis and treatment of intracellular bacteria. The daily scientific program was brightened up by highly didactic and interesting clinical quizzes. Every day, participants were able to interact thanks to evening extra-activities such as ping-pong, swimming and running. Not less worthy was the Thursday afternoon, spent in mountains surrounding Villars-sur-Ollon. The next postgraduate

technical workshop organised by ESCAR will be on the practical diagnosis of arthropod-borne infections, and will take place in Marseille (France) from 17th to 19th of March 2014. For sure, this will be another unique occasion to familiarize with intracellular bacteria, their intriguing biology and the important diseases they cause.

9) Acknowledgements

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Figure legend

Figure 1: Participants and venue. The top part is on overview of the hiking in the Swiss pastures. On the bottom left, the small but motivated group of early risers joggers, and on the bottom right, the friendly atmosphere that was present during lunch and diner.

Figure 2: Talks and speakers. On the top left, the beautiful presentation room with attentive participants, and three of the speakers, (i) Dr. M. Puolakainen (Helsinki, Finland), (ii) Dr. P.E. Fournier (Marseille, France), and (iii) Dr. D. Baud (Lausanne, Switzerland).

Figure 3: Practicals. An overview of the nine practicals organized in the Institute of microbiology of the University Hospital Center of Lausanne (IMUL-CHUV). From top to the bottom and left to the right: (1) dissection of ticks, (2) *Listeria*, phenotypes and phenotypic identification in the diagnostic laboratory, (3) amoebal enrichment, (4) immunofluorescence, (5) amoebal co-culture, (6) cell culture: isolation of bone marrow-derived macrophages, (7) doing a Gram and Diff-Quick staining, (8) bioinformatics for dummies: how to assemble a genome and how to annotate a genome, and (9) MALDI-TOF identification of strict intracellular bacteria: the *Chlamydiales* example.

Figure 1

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Figure 2

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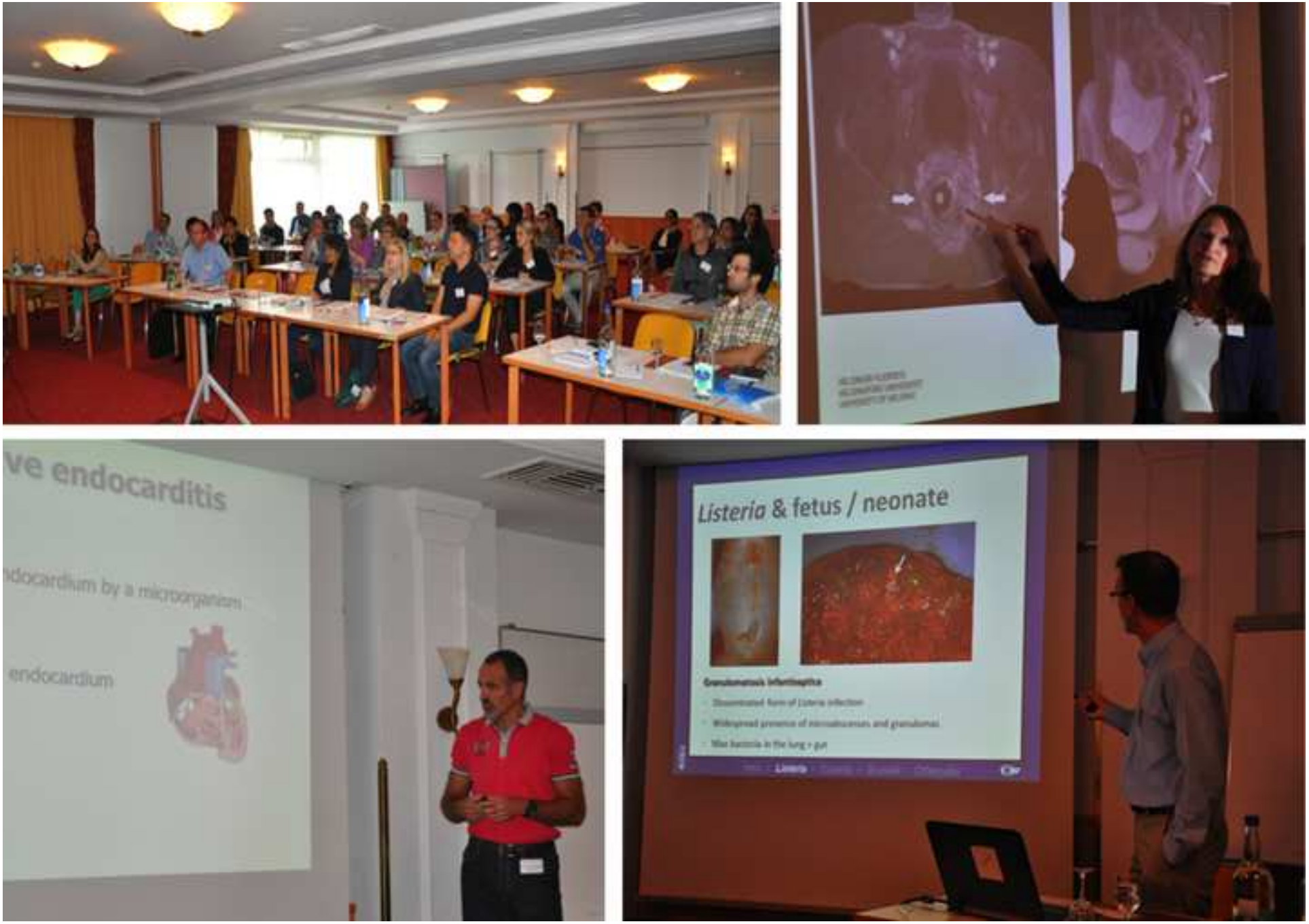

Figure 3

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