Romanian Journal of Morphology and Embryology 2006, 47(1):15–19

ORIGINAL PAPER

The HOPE technique opens up a multitude of new possibilities in pathology

E. VOLLMER, J. GALLE, D. S. LANG, S. LOESCHKE, H. SCHULTZ, T. GOLDMANN

Clinical & Experimental Pathology, Research Center Borstel, Germany

Abstract

Fixation of tissues with formalin results in well-preserved morphology but to a high degree leads to degradation of nucleic acids, which substantially constricts the spectrum of applicable molecular techniques. The novel HOPE-fixative with subsequent paraffin embedding, as an alternative to formalin, has been shown to result in a morphological preservation comparable to formalin-fixed, paraffin-embedded specimens. Due to a similar workflow like in formalin-fixation and paraffin embedding, the HOPE technique can be successfully established within any pathological institute. We have shown that DNA, RNA and proteins are protected in HOPE-fixed, paraffin-embedded tissues for at least eight years. Moreover, we described procedures which permit successful application of all common molecular techniques such as *in situ* hybridization targeting either DNA or RNA, immunohistochemistry without antigen retrieval and for formalin-refractory antigens, PCR, RT-PCR, Western blot, Northern blot, and transcription microarrays to HOPE-fixed, paraffin-embedded tissues. Furthermore, HOPE-fixed tissues can be used for the construction of tissue microarrays for enhanced high-throughput analyses on the molecular level. Using the HOPE technique as its crucial methodological base, *ex vivo* model systems could be established, e.g. for the simulation of early events in human infections and detection of chemotherapy resistances in human cancer. In addition to tissues, cell-culture preparations have been prepared utilizing the HOPE technique, which were then successfully applied to *in situ* hybridization targeting mRNA or immunocytochemistry with excellent preservation of morphological details. Taken together, the HOPE technique to date represents an alternative fixation that is, in contrary to other procedures, scientifically broadly analyzed. Therefore new possibilities are opened up especially within the rapidly growing field of molecular pathology.

Keywords: Hepes-glutamic-acid-buffer-mediated-Organic-solvent-Protection-Effect, tissue fixation.

Introduction and General Remarks

Any fixation of tissues produces artefacts. A long time ago Hippokrates discussed the biologic effects of mercury and alcohols as fixatives already in 400 B.C. With the invention of the microscope more attention was given to histological structures.

Systematic studies of the fixatives used for such purposes began not until the second half of the 19th century. These fixatives mainly served for the structural preservation.

Fixation with formalin and subsequent embedding in paraffin is well suited for this purpose and became a standard method. Since the development of monoclonal antibodies and their widespread use within diagnostics from the 1980s specific antigens have become detectable in tissue slices. The effect of fixation on the detection of antigens has been intensely discussed since then. Formalin fixed paraffin embedded (FFPE) material can be used for such immunohistochemical techniques only with limitations. A limited portion of antigenic epitopes is accessible for antibodies in FFPE.

Therefore, the panel of antibodies available for the use in FFPE is substantially smaller than that available for fresh or frozen specimens, the latter resulting in a comparatively inferior morphology. This can be compensated only partly by the development of highly sensitive immunohistochemical techniques in combination with antigen retrieval techniques for FFPE.

By the advent of biotechnologic techniques such as microarrays, proteomics, tissue arrays, etc., molecular diagnostics began to change the traditional workflow in pathology. As a prerequisite for molecular diagnostics the quality of nucleic acids and proteins within the specimens is becoming more and more important. FFPE materials constrict the application of molecular techniques since the preservation of nucleic acids and proteins is comparably low.

One actual example for such a need of molecular diagnostics is the therapy of breast cancer with the humanized anti-Her2 antibody herceptin (trastuzumab). For successful medication of the patients with this innovative drug adequate diagnostic procedures are necessary, which either test the overexpression of the Her2-protein by immunohistochemistry, or the amplification of the Her2-gene by fluorescent *in situ* hybridization (FISH). These diagnostic procedures might be problematic with FFPE materials due to the low preservation of proteins and nucleic acids, which is why intense discussions have been raised in order to reach at least some standardization. However, this standardization of the detection procedures applied to FFPE materials could not raise the quality of nucleic acids and proteins and is therefore of limited help and perspective. Beyond this, the fixation itself should be highly standardized too. The increasing demand for such molecular procedures, for example with regard to other members of the EGF-R family like Her1,

detection of Her2/Her3 heterodimers, etc., unavoidably leads to the need of an adequate fixation of the tissues. Another aspect is scientific projects, which also at least partly are constricted by the low preservation of proteins and nucleic acids in FFPE-materials. Utilization of frozen tissues is only a partial substitute mainly due to the low morphologic quality. An alternative procedure should ensure three objectives: preservation of nucleic acids and proteins, preservation of morphologic details and a high level of standardization.

Considering these aspects a fixation procedure which preserves nucleic acids facilitating the application of molecular techniques such as RT-PCR, DNA and RNA *in situ* hybridization, Northern blot, or microarrays, will be of high value for modern pathology in diagnostics as well as scientific research.

Applications and Experiences

The HOPE technique is based on a novel hyperosmolar protection solution composed of amino acids and organic buffers. Acetone is the only dehydrating agent followed by pure paraffin [1]. Therefore working conditions are very similar to FFPE. It has been shown that high molecular DNA and RNA can be extracted from HOPE-fixed tissues [2–4]. A result of an RT PCR-experiment using RNA from HOPE-fixed, paraffin-embedded lung tissues is exemplified in Figure 1.

Experiments using RNA from FFPE tissues work on residues of RNA, which do not necessarily resemble the natural situation of the original RNA population, found in the native tissue. We therefore developed a procedure, which enables RNA *in situ* hybridization experiments in HOPE-fixed materials [3]. The procedure utilizes randomly primed double stranded digoxygenated DNA-probes and due to the omission of signal enhancement the protocol is relatively short. This technique was successfully applied in several studies targeting even weakly transcribed molecules such as interleukins, etc. [5–17].

Preservation of morphologic details is good, as is the generation of unambiguously detectable signals. Negative controls remained negative as controls with irrelevant probes. These studies were verified by RT-PCR experiments throughout. Due to its high reliability all other signal enhancing procedures such as catalyzed reporter deposition (CARD) or *in situ* PCR have now become dispensable [3]. It is possible to apply this technique to paraffin blocks, which are eight years old; that is the time-span we have collected HOPE-fixed materials yet.

Immunohistochemical assessment of biological parameters has become an integral part of pathology. Cell proliferation is often measured in tumors and the determination of hormone receptors is mandatory in the reporting of breast cancer. Finally, there are FDA-approved immunohistochemical assays, which are decisive for adjuvant therapy, e.g. the HercepTest™. To our knowledge, however, there are no assay systems available applicable to routinely formalin-fixed and paraffin embedded material without antigen retrieval, which often leads to a loss in the morphological quality and the repeated heating and cooling are difficult to standardize. It is worthwhile to mention that concerning FFPE the tissue preparation steps up to the paraffin block are not standardized at all concerning e.g. the concentration of formalin, the fixation time etc. Therefore, we analyzed the immunohistochemical assessment of cell proliferation using the common MIB-1 antibody against the Ki-67 antigen, the detectability of estrogen- and progesterone-receptors and of FDA-approved assays we applied the HercepTest™ [18]. Formalin-fixed and correspondingly HOPE-fixed paraffin embedded tissues of mammary carcinoma specimens were investigated. The assessment of all these important biomarkers in paraffin sections requires cooking when formalin-fixation is used, whereas we achieved excellent immunostainings for these biomarkers investigated in HOPE-fixed paraffin sections without antigen retrieval [18].

Quantity and intensity of positively stained cells there was almost equal comparing the established protocols for FFPE using antigen retrieval and the HOPE-protocol without the latter. Moreover, the HOPE-fixed specimens required only 1/3rd of the primary antibodies as well as 1/3rd of the respective detection system.

The formalin-fixed specimens without antigen retrieval remained staining negative. Immunohistochemistry with HOPE-fixed materials to date has been utilized in numerous studies [6, 8, 17, 19, 20] in different tissues including even fragile tissues like vitrectomy specimens [21] in both animal and human tissues. Two examples of immunohistochemical results without antigen retrieval obtained in HOPE-fixed tissues using antibodies with diagnostic and therapeutic relevance respectively which both require antigen retrieval in FFPE-tissues are displayed in Figure 2 (Ki-67) and Figure 3 (EGF-R).

Due to the excellent preservation of antigens, highly standardized tissue sampling and the well-preserved morphology the HOPE technique fulfill the increasing demands in the expanding field of molecular diagnostics. The application of the HOPE technique for cytospin preparations of cultured cells showed that immunocytochemistry and RNA-*in situ* hybridization can be performed with these specimens after development of a suitable modified fixation protocol. Preservation of morphology was eminent as was the generation of signals [17]. Currently a technique is under development to endure HOPE-fixation and paraffin embedding with broncho-alveolar-lavage specimens in order to enhance the capabilities available to date in pneumology.

As a subsequent element of a comprehensive panel of molecular techniques we developed protocols for Western blots with HOPE-fixed tissues [22]. In a comparison of FFPE, frozen material and HOPE-fixed tissues it turned out that the detection of epitopes was equal in HOPE-fixed and frozen samples, while FFPE-tissues could not successfully be used for Western blot. The protocol used contains only slight modifications in comparison to the one for frozen materials.

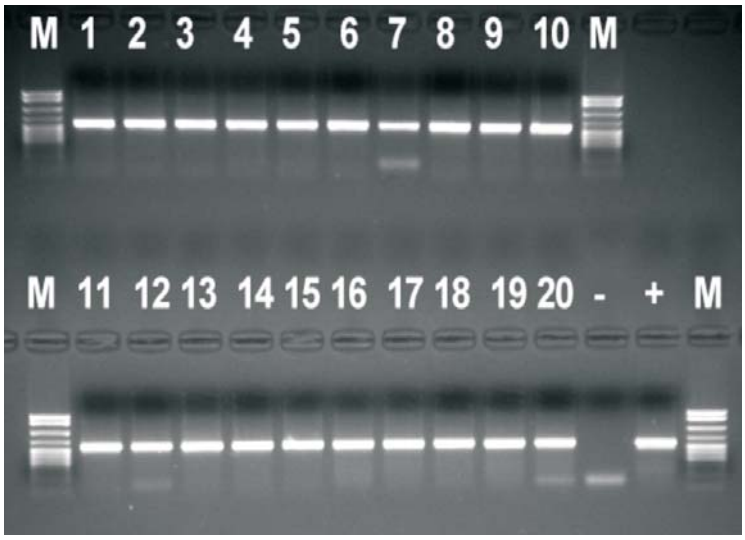

Figure 1 – *Photograph of an agarose gel electrophoresis separating products from an RT-PCR reaction using cDNA synthesized from total RNA extracted from human lung cancer tissue specimens that were previously HOPE-fixed and paraffin embedded (½). RT-PCR was targeting a 247 bp fragment of human glyceraldehyde-3-phosphate dehydrogenase transcript. Lanes ⅓ 6, ⅓¼ and ⅙ show directly HOPE-fixed samples, the other lanes (2–5, 7–⅓0, ½2–⅓5, ⅓7–20) are samples from according tissues which had been stimulated in the STST ex vivo model for ⅙ hours and HOPE-fixed afterwards: (–) – negative control without template; (+) – positive control from frozen tissue. The molecular weight marker (M) used with these 2% agarose gels was pBR 322-Msp½*

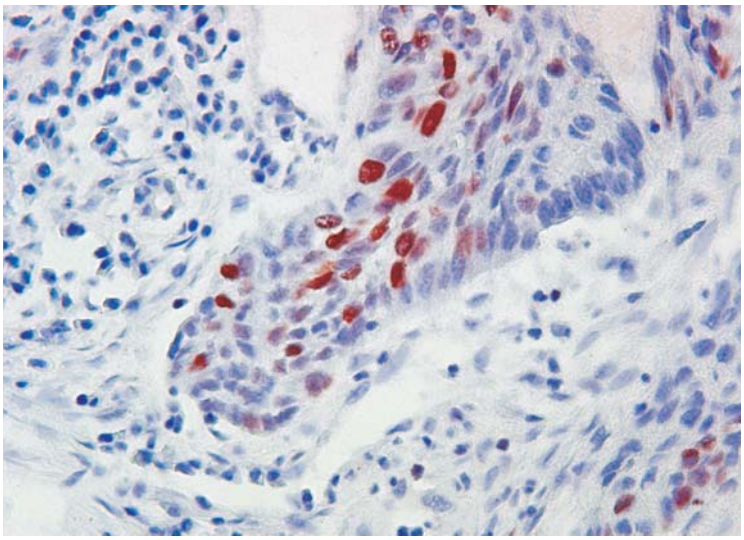

Figure 2 – *Photomicrograph (×400) of an immunohistochemical detection of Ki-67 in human non-small cell lung cancer tissues. This experiment was performed by the use of tissue microarrays from HOPE-fixed specimens. MIB-½antibody was used like described above together with the LSAB procedure without any antigen retrieval*

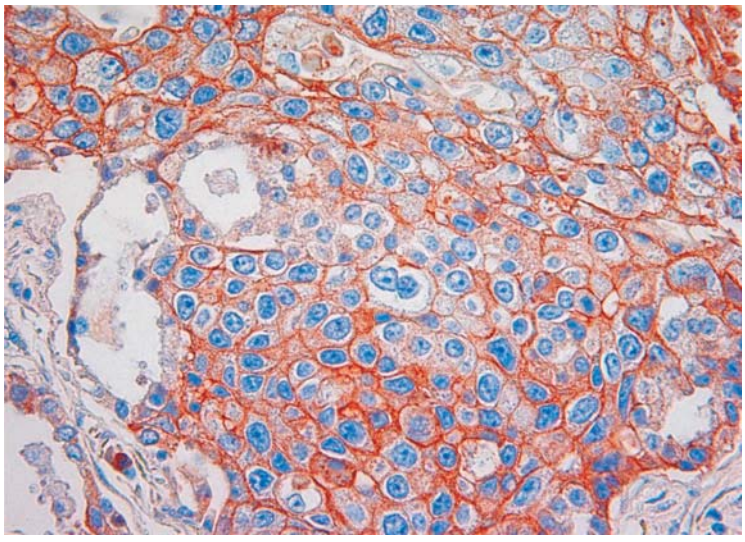

Figure 3 – *Photomicrograph (×400) of an immuno-histochemical detection of Epithelial Growth Factor-Receptor (EGF-R) in human non-small cell lung cancer tissues using tissue microarrays from HOPE-fixed specimens. Anti EGF-R antibody clone 2-½Æ½ (Zytomed Systems, Germany) received as a ready to use antibody was diluted in Tris-buffer and applied for one hour in a moist chamber without any antigen retrieval. Detection was achieved by application of the LSAB procedure*

To further extend the panel of techniques on the RNA-level we showed that HOPE-fixed tissues could be used for Northern blot-hybridization and transcription microarrays [2].

The Northern blot hybridization targeted two splice variants of human HMGB1 (1.4 kb and 2.4 kb) in total RNA extracted from HOPE-fixed breast cancer tissues, which both were unambiguously detected as was GAPDH. RNA integrity as judged on gel was high with clear 18S and 28S rRNA bands visible on the image. The same holds true for transcription microarray, which also displayed to well preserved RNA in HOPE-fixed tissues and was carried out without any specialized protocol.

Due to the high molecular weight DNA extractable from HOPE-fixed materials we were able to demonstrate by utilizing real time RT-PCR that the detection of infectious agents like *Mycobacterium tuberculosis* is clearly enhanced with an at least 100-fold increased sensitivity if comparing HOPE-fixed tissues to FFPE [16].

Moreover subtyping of the mycobacteria was clearly achieved in HOPE-fixed samples by spoligotyping, which was not successful with FFPE. HOPE-fixed materials were used for quality control in a German reference testing of PCR targeting mycobacteria [23].

According to the detection of infectious agents a methodological similar approach like the one used for RNA *in situ* hybridization was developed for DNA *in situ* hybridization and successfully applied for the detection of *Chlamydiae* in HOPE-fixed tissues [24].

This *in situ* hybridization was found to display higher sensitivity than PCR from the same materials although being highly specific. Partly, this work has been performed using an ex vivo infection model with human lung tissues. This way of cultivating human lung tissues for short term with subsequent HOPE-fixation has been initially described during the development of the RNA *in situ* hybridization procedure [3].

It was designated Short Term Stimulation of Tissues (STST). In this functional human model comparatively small tissue samples (approx. 0.5 g) are being cultivated for 16 hours with and without various stimuli. After HOPE-fixation the effects of such stimulation experiments can be analyzed on the molecular level, and, moreover, these analyses can be performed *in situ*. Besides infectious processes this model has served for studying the effects of the treatment of human non-small cell lung cancer tissues with bacterial oligonucleotides (CpG) [6]. This combination of human tissue culture and the HOPE technique with its widespread possibilities for read out serves as our central model in the investigation of lung diseases.

We recently showed that laser microdissection and pressure catapulting (LMPC) could be performed with HOPE-fixed tissues [25]. This study also describes a protocol for downstream real time RT-PCR, which showed the clearly superior results, obtained with HOPE-fixed specimens in comparison to FFPE. These RT-PCR analyses were performed without any RNA amplification procedure. This results in higher specificity and reproducibility if compared to protocols, which have to use such techniques due to degradation within FFPE specimens.

Tissue microarrays have been constructed from HOPE-fixed tissues using the MTA1 tissue microarray device (Alphametrix, Rodgau, Germany) and it turned out that all molecular techniques described above for HOPE-fixed sections can also be applied to these tissue microarrays. This enables high throughput molecular studies in paraffin embedded tissues [26, 27]. Two examples of immunohistochemical stainings with tissue microarrays of HOPE-fixed tissues are shown in Figures 2 and 3.

Conclusions

Taken together, the HOPE technique enables the successful application of all common molecular techniques with enormously enhanced results when compared to FFPE. Moreover, the HOPE technique is highly standardized and leads to high inter specimen comparability. An automated system for HOPE-fixation and paraffin embedding will be available in 2006. HOPE fixed specimens display an excellent formalin-like morphology.

We conclude that the HOPE technique is an ideal alternative fixation for modern pathology.

Acknowledgements

The authors like to thank H. Kühl, W. Martens, J. Tiebach, J. Hofmeister, D. Kähler, T. Zietz, and D. Bubritzki for their technical attendance.

References

[1] OLERT J., WIEDORN K. H., GOLDMANN T. *et al.*, *HOPE-fixation: A novel fixing method and paraffin embedding technique for human soft tissues*, Pathol Res Pract, 2001, 197:823–826.

[2] GOLDMANN T., FLOHR A. M., ESCOBAR H. M. *et al.*, *The HOPE technique permits Northern blot and microarray analysis in paraffin-embedded tissues,* Pathol Res Pract, 2004, 200(7–8):511–515.

[3] GOLDMANN T., WIEDORN K. H., KÜHL H. *et al.*, *Assessment of transcriptional gene activity in situ by application of HOPE-fixed, paraffin-embedded tissues*, Pathol Res Pract, 2002, 198:91–95.

[4] WIEDORN K. H., OLERT J., STACY R. *et al.*, *Preservation of high molecular weight nucleic acids by application of the novel HOPE-fixative*, Pathol Res Pract, 2002, 198:735–740.

[5] BLUMENTHAL A., EHLERS S., LAUBER J. *et al.*, *Human Wnt5a bridges innate and adaptive immune responses to microbial stimuli*, Blood (in press).

[6] DROEMANN D., ALBRECHT D., GERDES J. *et al.*, *Human cancer cells express functionally active Toll-like receptor 9*, Resp Res, 2005, 6(1):1.

[7] DROEMANN D., GOLDMANN T., TIEDJE T. *et al.*, *Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients*, Respir Res, 2005, 6(1):68.

[8] DROEMANN D., GOLDMANN T., BRANSCHEID D. *et al.*, *Toll-like Receptor 2 (TLR2) is expressed by alveolar type 2 cells and macrophages in the human lung*, Histochem Cell Biol, 2003, 119:103–108.

[9] FRICKE I., MITCHELL D., MITTELSTÄDT J. *et al.*, *Mycobacteria induce IFN-gamma production in human dendritic cells via triggering of toll-like receptor 2*, J Immunol, 2006, 176(9):5173–5182.

[10] KUEBLER W. M., UHLIG U., GOLDMANN T. *et al.*, *Stretch activates nitric oxide production in pulmonary vascular endothelial cells in situ*, Am J Respir Crit Care Med, 2003, 168:1391–1398.

[11] PECHKOVSKY D., GOLDMANN T., LUDWIG C. *et al.*, *CCR2 and CXCR3 agonistic chemokines are differently expressed and regulated in human alveolar epithelial cells type II*, Resp Res, 2005, 6(1):75.
[12] PECHKOVSKY D., ZISSEL G., GOLDMANN T. *et al.*, *Different Pattern of NOS2 and NOS3 mRNA expression in human A549 cells and primary cultured alveolar epithelial cells type II*, Am J Physiol, 2002, 282:L684–692.
[13] PECHKOVSKY D., ZISSEL G., STAMME C. *et al.*, *Human alveolar epithelial cells type synthase-2 expression in alveolar macrophages by an interferon-gamma-depended pathway II induce nitric oxide*, Eur Resp J, 2002, 19(4):672–683.
[14] PECHKOVSKY D. V., GOLDMANN T., VOLLMER E. *et al.*, *Interleukin-18 expression by alveolar epithelial cells type II in tuberculosis and sarcoidosis*, FEMS Immunol Med Microbiol, 2006, 46(1):30–38.
[15] PRASSE A., PECHKOVSKY D. V., TOEWS G. B. *et al.*, A v*icious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis via CCL18*, Am J Respir Crit Care Med, 2006, 173:781–792.
[16] UHLIG U., FEHRENBACH H., LACHMANN R. A. *et al.*, *Phosphoinositide 3-OH kinase inhibition prevents ventilation-induced lung cell activation*, Am J Respir Crit Care Med, 2004, 169:201–208.
[17] UMLAND O., ULMER A. J., VOLLMER E., GOLDMANN T., *In situ hybridization and immunocytochemistry using HOPE-fixed, cultured human cells on cytospin preparations*, J Histochem Cytochem, 2003, 51:977–980.
[18] GOLDMANN T., VOLLMER E., GERDES J., *What's cooking? Detection of important biomarkers in HOPE-fixed paraffin embedded tissues eliminates the need for antigen retrieval*, Am J Pathol, 2003, 163:2638–2640.
[19] BUSCHE A., GOLDMANN T., NAUMANN U. *et al.*, *Natural killer cell-mediated rejection of experimental human lung cancer by genetic overexpression of major histocompatibility complex class I chain-related gene a*, Hum Gene Ther, 2006, 17(2):135–146.
[20] UHLIG U., HAITSMA J. J., GOLDMANN T. *et al.*, *Ventilation-induced activation of the mitogen activated proteinkinase pathway*, Eur Respir J, 2002, 20:946–956.
[21] COUPLAND S. E., PEREZ-CANTO A., HUMMEL M. *et al.*, *Assessment of HOPE fixation in vitrectomy specimens in patients with chronic bilateral uveitis (masquerade syndrome)*, Graefes Arch Clin Exp Ophthalmol, 2005, 243(9):847–852.
[22] UHLIG U., UHLIG S., BRANSCHEID D. *et al.*, *HOPE technique enables Western blot analysis from paraffin-embedded tissues*, Pathol Res Pract, 2004, 200(6):469–472.
[23] SEN GUPTA R. D., HILLEMANN T., KUBICA G. *et al.*, *HOPE-fixation enables improved PCR-based detection and differentiation of Mycobacterium tuberculosis complex in paraffin-embedded tissues*, Pathol Res Pract, 2003, 199:619–623.
[24] SCHEWE C., GOLDMANN T., GROSSER M. *et al.*, *Inter-laboratory validation of PCR-based detection of Mycobacterium tuberculosis in formalin-fixed, paraffin-embedded tissues*, Virchows Arch, 2005, 447(3):573–585.
[25] GOLDMANN T., BURGEMEISTER R., SAUER U. *et al.*, *Enhanced molecular analyses by combination of the HOPE technique and laser microdissection*, Diagn Pathol, 2006, 1(1):2.
[26] GOLDMANN T., DRÖMANN D., MARZOUKI M. *et al.*, *Tissue microarrays from HOPE-fixed specimens allow for enhanced high throughput molecular analyses in paraffin-embedded material*, Pathol Res Pract, 2005, 201:599–602.
[27] RUPP J., DROEMANN D., GOLDMANN T. *et al.*, *Alveolar epithelial cells type II are major target cells for C. pneumoniae in chronic but not in acute respiratory infection*, FEMS Immunol Med Microbiol, 2004, 41:197–203.

Mailing address
Ekkehard Vollmer, Professor, M. D., Ph. D., Clinical & Experimental Pathology, Research Center Borstel, Germany; E-mail: evollmer@fz-borstel.de

Received: July 6th, 2006

Accepted: July 20th, 2006