IKDT GmbH, Moltkestrasse 31, D – 12203 Berlin

IKDT Laboratory

Your Partner for Molecular Diagnostics in Primary and Secondary Cardiomyopathies

IKDT GmbH, Moltkestrasse 31, D – 12203 Berlin

IKDT Portfolio 2021

Contents

IKDT GmbH, Moltkestrasse 31, D – 12203 Berlin

Managing Director/CEO
Prof. Dr. Heinz-Peter Schultheiss, MD

Laboratory Director/ Managing Director
Dr. Ganna Aleshcheva, PhD

Medical Director
Prof. Dr. Ulrich M. Gross, MD

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1. A Brief History

Since its foundation in 2002, the Institute for Cardiac Diagnostics and Therapy (IKDT) has become one of the leading diagnostic centres for viral infections and inflammation of heart muscle tissue. Using the most advanced analytical devices, our well-trained and experienced medical technical staff process patients and research samples and perform molecular diagnostics.
In close cooperation with the Department of Cardiology and Pulmonology at the Charité Medical Clinic II in Berlin, IKDT routinely performs standard as well as specialized diagnostics of cardiologic diseases. Almost any requested endomyocardial biopsy (EMB) procedure can be performed here in accordance with international recommendations and guidelines. Our services are used by cardiological hospitals throughout Germany, Europe and worldwide.
IKDT is the only clinical laboratory in Germany accredited by the *College of American Pathologists (CAP)* to perform extended diagnostics of endomyocardial biopsies. The implemented QM system meets the GLP/GCP guidelines and Clinical Laboratory Improvement Amendments of 1988 (CLIA-88).

2. Our Service

IKDT performs diagnostics on EMBs for hospital-affiliated institutions or private medical practices. Routine diagnostics covers three main areas:
Histology
Immunohistochemistry
Molecular Biology
Molecular Virology

Six EMBs at minimum are required for the routine procedure. For diagnostic accuracy, an increased number of EMBs (up to 9) is beneficial.

Histological and immunohistochemical examination is completed within two working days; molecular virology in 3-4 days: thus routine diagnostics of EMBs usually takes five days.

3. Quality Assurance and Quality Management

From the beginning, our in-house quality management (QM) system was informed by the College of American Pathologists (CAP) Laboratory Accreditation Program (LAP). Established in 1961, CAP was approved in 1995 as an accrediting organization under the Clinical Laboratory Improvement Amendments of 1988 by the Centres for Medicare and Medicaid Services, an agency of the US Department of Health and Human Services. CAP is the only organization approved by the US Food and Drug Administration (FDA) to accredit diagnostic labs outside the US. Laboratories that meet their accreditation requirements distinguish themselves as quality assured.

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The Laboratory Accreditation Program includes a biannual on-site peer inspection of the laboratory by two or three inspectors, as well as intervening self-inspection by the laboratory director. IKDT has successfully achieved LAP accreditation to perform endomyocardial biopsy diagnostics under certified conditions for the eighth time since 2003 (Figure 1).
LAP entails an ongoing commitment to improving the quality of clinical laboratory services through voluntary participation, peer review, education and compliance with established performance standards. The objective is to achieve efficient and timely sample processing while ensuring a permanent high quality standard of data acquisition by means of state-of-the-art diagnostic methods, supplemented by detailed data reporting. IKDT has established a rigorous sample and data management system, ensuring full traceability of any sample at any time and enabling the laboratory to respond quickly to undefined deviations.


COLLEGE of AMERICAN PATHOLOGISTS

CAP ACCREDITED

The College of American Pathologists
certifies that the laboratory named below

Institut Kardiale Diagnostik und Therapie GmbH
Laboratory
Berlin, Germany
Ganna Aleshcheva, PhD

CAP Number: 7182802
AU-ID: 1397839

has met all applicable standards for accreditation and is hereby accredited by the College of American Pathologists' Laboratory Accreditation Program. Reinspection should occur prior to June 20, 2021 to maintain accreditation.

Accreditation does not automatically survive a change in director, ownership, or location and assumes that all interim requirements are met.

Chair, Accreditation Committee

President, College of American Pathologists

Figure 1 Certificate 2019 of IKDT lab accreditation by College of American Pathologists (CAP)

LAP is the gold standard for laboratory accreditation and the only institution fulfilling all the requirements of US, European and German Quality Management Boards (QMBs) (Figure 2). The IKDT QM system follows internationally accepted GLP/GCP CLIA-88 guidelines, and our laboratory director and members of the consulting board (Charité University Hospital) ensure the quality of examination results.

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Accreditation programs examine pre-analytical, analytical and post-analytical aspects of quality management in the laboratory. This includes the performance and monitoring of general quality control (QC), test methodologies and specifications, reagents, controls and media, equipment, specimen handling, test reporting and internal performance assessment and external proficiency testing. In addition, personnel requirements, safety, document management and other management practices are included in the inspection process.

IKDT has all the necessary space, facilities and personnel to meet these high standards, with advanced equipment checked regularly by maintenance contractors. Explicit standard operating procedures (SOPs) apply to all equipment and methods, and IKDT's highly qualified and capable personnel constantly update their skills and knowledge through continuing medical education.
PCR results are the most critical part of molecular analysis, as they determine decisions about clinical treatment and therapy. Detection of virus infection in EMBs is a multi-step procedure, and each step is essential to the final result. At IKDT, ongoing controls monitor, prove and validate all PCR results.

AQS - Baden-Württemberg
Analytische Qualitätssicherung Baden-Württemberg am Institut für Siedlungswasserbau, Wassergüte- und Abfallwirtschaft (ISWA) der Universität Stuttgart
Hauptaufgabe der AQS sind Ringversuche im Bereich der Wasseranalytik (Liste von Ringversuchen, Download von Ringversuchsauswertungen) - AQS-Kurse (z.B. Probenahme von Abwasser) - Linksammlung

CAP - College of American Pathologists: Laboratory Improvement/Practice
Besonders ist das CAP Laboratory Improvement Program - US-Standard ("gold standard") zur Akkreditierung medizinischer Laboratorien - Liste der CAP akkreditierten Laboratorien, CAP Programm zur Laborakkreditierung, diverse Checklisten zur CAP-Akkreditierung (Word, HTML und PDF), Pathology Practice Parameters and Guidelines, News, kategorisierte Linkliste

CITAC - Cooperation on International Traceability in Analytical Chemistry
hat sich zum Ziel gesetzt, die internationale Vergleichbarkeit chemischer Analysen in Zusammenarbeit mit anderen Organisationen zu fördern. Newsletter (1xjährlich), Kontaktadressen, ein paar Links

DAR - Deutscher Akkreditierungsrat
Die übergeordnete deutsche Stelle für die Akkreditierung von Zertifzierungsstellen, Akkreditierungsstellen. Sehr informative Seite mit vielen Dokumenten zu verschiedenen Topics der Akkreditierung von Labors, Listung und Datenbank (kaum zu bedienen) der akkreditierten Labors gegliedert z. B. nach Bundesland oder akkreditiertem Bereich usw. - Das Layout ist eher anspruchslos.

EA - European cooperation for Accreditation
entstanden aus EAC (European Accreditation of Certification) and EAL (European co-operation for Accreditation of Laboratories) entstanden ist. Die EA deckt in Europa übergeordnet alle Aktivitäten der Konfomitätsbewertung für Prüfung und Kalibrierung, Inspektion, zertifizierung von QM-Systemen, Produkten und Personal sowie die EMAS-Verifizierung (Umwelt) ab.
u.a. Liste aller Mitgliedsorganisationen, EA-Dokumente als PDF-Files

Eurachem
Internationale Rückführbarkeit bzw. Rückverfolgbarkeit chemischer Analysen ist hier der Focus
Newletter - Dokumente (meist kostenpflichtig) - nationale Organisationen - Links

EUROLAB-D - Verein Deutscher Prüflaboratorien e.V.
Vertritt deutsche analytische Laboratorien - Die Seite bietet lediglich Angaben zu Organisation, Ansprechpartnern und Workshop-Proceedings

IAF - International Accreditation Forum, Inc.
Die IAF ist eine internationale Vereinigung, die sich mit der Konformitätsbewertung von Akkreditierungsstellen und anderen Bodies befasst und ein weltweit einheitliches Systems zur Konformitätsbewertung solcher Stellen anstrebt. Einige Bereiche der Site nur für Mitgliedern zugänglich!

ILAC- International Laboratory Accreditation Cooperation
News (ca. alle 6 Monate) und Guidelines / Documents (PDF-Dateien) zu Laborakkreditierung, Qualitätssicherung,

Figure 2 Extract from Ranking of CAP Laboratory Improvement Program by German Quality Management Board (Source: www.qmb.de, 2000).

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IKDT participates twice each year in national surveys on nucleic acid amplification techniques (NAT) for virus detection, organized by INSTAND e.V., Düsseldorf (Germany). INSTAND provides individual laboratories with unknown specimens for testing. The participants analyse the specimens and return the results to INSTAND for evaluation. In turn, each participating laboratory receives a report on their performance and a certificate attesting successful participation.

In an additional report, the results of all participating laboratories are summarized. IKDT uses these surveys to evaluate and benchmark its own in-house testing. To date, IKDT has always participated successfully (see Figure 3).

INSTAND

ZERTIFIKAT

Ringversuch vom 27.11.2020

Sie haben die Anforderungen des Ringversuchs mit den folgenden Untersuchungen erfüllt

Virusgenom-Nachweis - Adenoviren (371):
Gültigkeitsdauer 12 Monate:
Adenoviren (DNA) - quantitativ (R: B3)
Adenoviren (DNA) - qualitativ (R: B3)
Adenoviren (DNA) - Spezies-Bestimmung (R: B3)
Adenoviren (DNA) - Typ-Bestimmung (R: B3)

Virusgenom-Nachweis - Cytomegalievirus Programm 1 (365):
Gültigkeitsdauer 12 Monate:
CMV (DNA) - qualitativ (R: B3)

Virusgenom-Nachweis - Enteroviren (372):
Gültigkeitsdauer 12 Monate:
Enteroviren (RNA) - quantitativ (R: B3)
Enteroviren (RNA) - qualitativ (R: B3)
Enteroviren (RNA) - Typ-Bestimmung (R: B3)

(R) diese Untersuchung unterliegt den RiliBÄK

Teilnehmer:
2285
Dr. Ganna Aleshcheva
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Düsseldorf, 22.12.2020

Prof. Dr. med. Michael Spannagl
(Leiter der Referenzinstitution)

Prof. Dr. rer. nat. Heinz Zeichhardt
(Ringversuchleiter)

INSTAND
Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e.V.
Ubierstr. 20 | 40223 Düsseldorf

2285 1 / 9

INSTAND

ZERTIFIKAT

Ringversuch vom 27.11.2020

Sie haben die Anforderungen des Ringversuchs mit den folgenden Untersuchungen erfüllt

Virusgenom-Nachweis - Epstein-Barr-Virus (376):
Gültigkeitsdauer 12 Monate:
EBV (DNA) - quantitativ (R: B3)
EBV (DNA) - qualitativ (R: B3)

Virusgenom-Nachweis - Herpes-simplex-Virus Typ 1/Typ 2 (363):
Gültigkeitsdauer 12 Monate:
HSV 1 (DNA) - quantitativ (R: B3)
HSV 1 (DNA) - qualitativ (R: B3)
HSV 2 (DNA) - qualitativ (R: B3)
HSV (DNA) - qualitativ ohne Differenzierung von HSV 1 und HSV 2 (R: B3)

Virusgenom-Nachweis - Parvovirus B19 (367):
Gültigkeitsdauer 12 Monate:
Parvovirus B19 (DNA) - quantitativ (R: B3)
Parvovirus B19 (DNA) - qualitativ (R: B3)

(R) diese Untersuchung unterliegt den RiliBÄK

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2285 2 / 9

Figure 3 Certificates of successful participation in the national INSTAND NAT survey

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4. IKDT Personnel

Laboratory Director Dr. Ganna Aleshcheva is a biotechnologist with lengthy experience in molecular biology.

Prof. Dr. Ulrich Gross, formerly Director of the Institute of Pathology at Charité University Hospital, is responsible as medical director for the entire laboratory and for histological examination of endomyocardial biopsies.

Prof. Dr. Heinz-Peter Schultheiss, CEO, former Director of the Medical Clinic II (Department of Cardiology) at Charité University Hospital, coordinates all medical and management activities.

As well as the medical and managing directors, IKDT currently employs five medical technical assistants, one biologist, one biotechnologist (PhD) and one secretary. Physician from the consulting board of the Department of Cardiology at Charité University Hospital and pathologists from DRK Kliniken Westend support the work of the IKDT laboratory, validating diagnostic findings, writing diagnostic reports and providing clinical recommendations for senders of EMBs.

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5. Pre-analytical treatment of patient material

IKDT performs diagnostic tests for detection of inflammatory processes or viral infection of human EMBs in stringent compliance with FDA guidelines. The diagnostic spectrum includes histology, immunhistochemical analysis and PCR assays for detection of viral genomes. Myocardial biopsies in fixed form are the preferred sample materials for these tests. For meaningful assays and results, it is important that the submitted material undergoes well-defined pre-analytic treatment (see Table 1).
For histology, EMBs should be fixed in buffered formalin (4-5%). For immunohistology and molecular biology EMBs should be stored in a novel reagent for conservation at ambient temperature. *RNAlater* is an aqueous, non-toxic tissue storage reagent that stabilizes and protects cellular RNA in intact, unfrozen tissue samples, eliminating the need to immediately process samples or freeze them in liquid nitrogen for later processing. IKDT provides senders with screw cap tubes filled with ***RNAlater*** for immediate use. Biopsies fixed in *RNAlater* should then be transferred to IKDT for diagnostic procedure as soon as possible.

RNAlater enables isolation of genomic DNA or RNA and immunohistochemistry. Tissue pieces can be harvested and submerged in *RNAlater* for storage without jeopardizing the quality or quantity of RNA obtained after subsequent RNA isolation. *RNAlater* can be added to cell pellets and even to cells in medium, and samples can then be stored, frozen or unfrozen.

Measuring less than 0.5 cm in any one dimension, EMBs are simply submerged in approximately 0.5 ml of *RNAlater* at room temperature by inverting the tube five times. The solution permeates the tissue or cells and stabilizes the RNA. Ideally, samples should be sent directly to IKDT lab at ambient temperature or stored at +4 °C before transport. Samples can be transported in a padded envelope by conventional mail. On arrival at the IKDT lab, the sample will be stored at +4 °C for one night and can then be stored at 4 °C for up to a month (at 25 °C for up to a week, and at -20 to -80 °C for an indefinite period). *RNAlater* is suitable only for very small tissue samples (i.e. biopsies). Larger pieces (explanted heart samples) must quickly be divided into smaller portions or cryo-conserved in liquid nitrogen.

Table 1 *Pre-analytical treatment of endomyocardial biopsies for diagnostics at IKDT*

Submitted material	**Pre-analytic treatment**	**Detection method**	**Native/ fixed**	**Shipment**
Myocardial biopsies	***RNAlater***	PCR, Immunohistochemistry	**Fixed in *RNAlater***	**Ambient temp**
Myocardial biopsies	Fixed in 4–5% buffered formalin	**Histology only**	Fixed in formalin	Ambient temp; **Do not freeze**

Important: ***Never freeze or treat biopsies with other fixatives before RNAlater fixation!***

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Additional analysis

Detection of systemic viral infections or cytokine profiles is performed by analysis of peripheral blood fractions. DNA or RNA from peripheral blood cells is examined by nested- and quantitative PCR (qPCR) for the presence of viral genomes, for characterization of viral activity and for exclusion or confirmation of systemic infection. EDTA blood is required for detection of systemic viral infection (Table 2).

Table 2 *Pre-analytical treatment of peripheral blood fractions for diagnostics at IKDT*

Submitted material	Pre-analytic treatment	Detection method	Native/ fixed	Shipment
Blood	EDTA-tubes	PCR	Native	+4 °C or ambient

6. Histology

For histological examinations, 4–5 µm-thick sections of paraffin-embedded biopsies are prepared by cutting with a rotary microtome. For each staining procedure, 3–8 serial sections are placed on one microscopic slide. Routine diagnostics always includes AZAN, HE, PAS and Elastica v. Weigert staining. With the exception of special stainings (Figure 4), staining of specimens is always carried out in the staining machines.

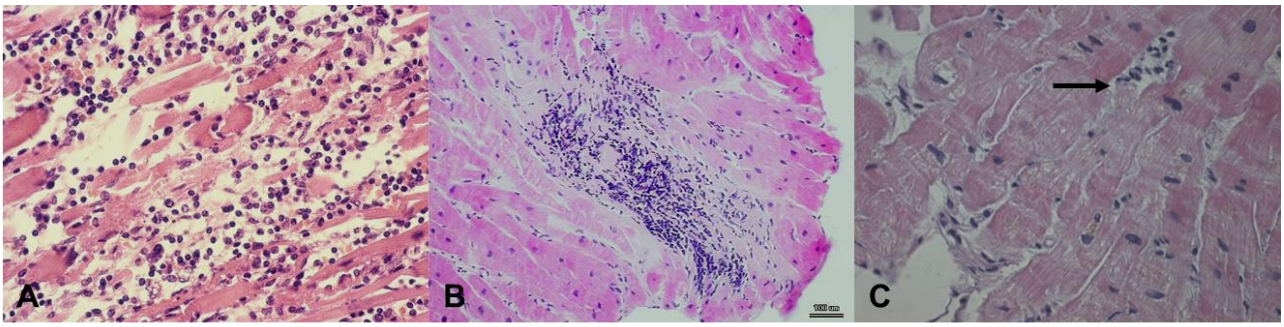

Figure 4 Histological presentation of acute myocarditis with diffuse (A) or focal infiltration of inflammatory cells (B), and focal infiltration with myocytolysis(see arrow) (C)

Special staining for amyloid (Congo red), calcium (v. Kossa), acid mucosubstances (Alcian Blue) and iron (Prussian Blue reaction) will be added in clinically suspected cases or at the customer´s request (Figure 5).
All histological examinations are performed by an anatomical pathologist. Histological examination in the IKDT lab follows the Dallas criteria for exclusion of acute or active myocarditis in examined biopsy samples. The main focus is on detection of myocytolysis in combination with leukocytic infiltrates.

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Observations are archived as printouts and as digitally printed colour photographs, which are saved and stored on the data server in TIFF or JPG format. Morphologic characteristics of stained endomyocardial tissue (e.g. diameter of cardiomyocytes, size and quality of biopsy, fibrosis, fatty tissue, capillaries) are rated by numeric scaling, and the corresponding values are documented in a written examination protocol and in the IKDT electronic database.

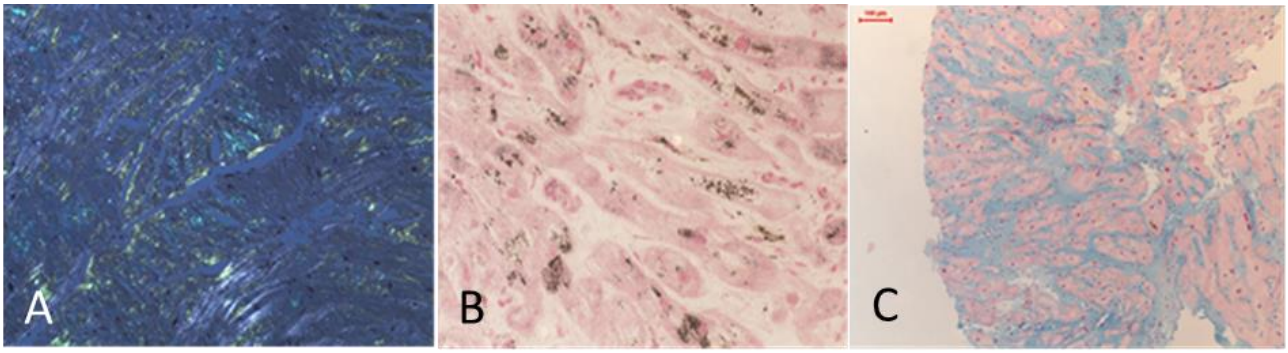

Figure 5 Special stains of amyloid with Congo Red visualized in polarized light (A), von Kossa stain for calcium (B) and Alcian Blue for acid mucosubstances (C).

7. Immunohistochemistry

Immunohistological diagnostics is based on the application of specific primary antibodies to cryo-fixed tissue sections and subsequent detection by a secondary antibody. The secondary antibody is conjugated with an enzyme complex, which produces a precipitating coloured complex after use of the staining solution.

For immunohistochemical examinations, tissue sections are prepared from cryo-embedded biopsies by use of a cryostat microtome. An endomyocardial biopsy will be placed on a pre-cooled (-20 °C) metallic tray and covered completely with the plastination glue Tissue-Tek. The glue freezes down immediately, preserving the embedded tissue at hard consistency.

For each antibody, cryo sections are usually prepared (about 20 cryo sections per patient) before immuno-staining commences. Finally, one microscopic slide ultimately contains separated areas for two different antibodies, with about 3-6 cryo sections in any area. This procedure and array design ensures more detailed analysis by simultaneous staining of different layers of biopsy by various antibodies.
Separated areas are then processed with different antibodies, accompanied by appropriate blocking and incubation steps and finally stained by an enzymatic conversion of the dye AEC to produce red-coloured immunospots for subsequent microscopic examination. The secondary antibody and colorimetric substrate are pre-mixed, having been thoroughly optimized for digital image analysis. The final counterstaining of cryo sections is always carried out in a staining machine (hematoxyline staining) (Figure 6).

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Three sets of immunohistochemical stainings are offered for specialized diagnostics of heart muscle tissue, whereas only the set IC1-*Heart muscle inflammation* is performed in the routine diagnostics procedure. Set IC2-*ARVD Diagnostics* is recommended in clinically suspected cases of ARVD, to detect disrupted gap junctions between cardiomyocytes. Set IC3-*Evaluation of myocardial fibrosis* is recommended for quantification and characterization of intramyocardial fibrosis.

IC1: ***Heart muscle inflammation*** (CD3, CD11a, CD11b, CD45RO, Perforin, CD54, CD106)
IC2: ***ARVD Diagnostics*** *(Plakoglobin, Connexin 43, N-Cadherin)*
IC3: ***Evaluation of myocardial fibrosis*** *(Collagen 1 and 3 incl. Sirius-Red staining)*

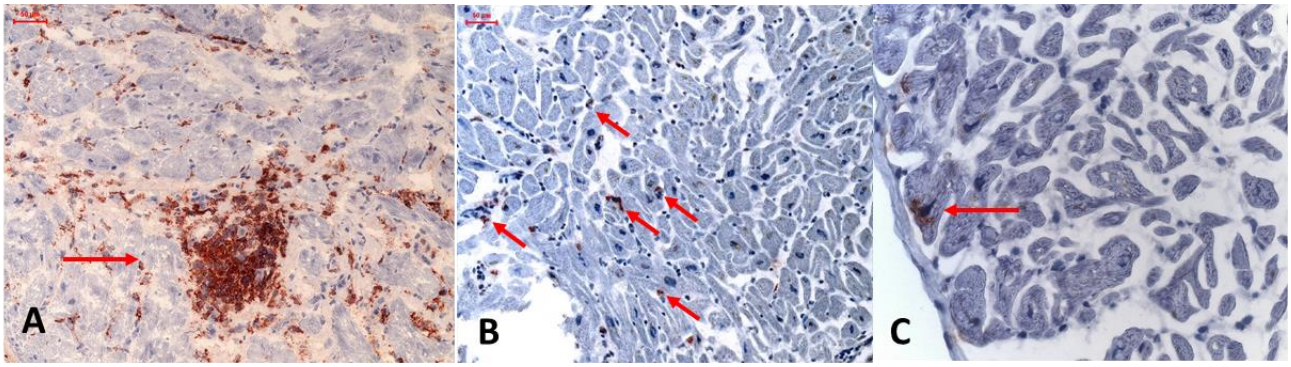

Figure 6 Immunostaining of massive focal infiltration by macrophages (A), diffuse infiltration by cytotoxic T-cells (Perforin) and detection of PVB19 by VP2 antibody (C) (see arrows).

Coloured immuno-spots are counted digitally by application of an in-house established digital imaging analysis software for calculating area fractions, numbers of immuno-spots and area of myocardial tissue (routine diagnostics). Values for counting are documented by the inclusion of digitally produced values in an electronic database for the final report, which also contains numeric values for morphological characteristics such as biopsy size, quality and fibrosis. Physicians at IKDT evaluate the digital results by additional examination and prepare the report for immunohistochemistry, including laser printed colour photographs for the sender. In general, immunohistochemical staining is performed on frozen sections of a second set of biopsies non-identical to the histological examination. This procedure is beneficial in reducing any possible misinterpretation that might be caused by limiting evaluation to a single biopsy.

Immunohistochemical analyses are carried out on frozen sections (two EMBs) to enable detection of elevated inflammatory cell subsets, including non-paraffin staining antibodies—e.g. CD3, CD11a (LFA-1), CD11b (MAC-1), CD45RO (memory or activated lymphocytes), perforin-positive cytotoxic lymphocytes and increased expression of the adhesion molecules CD54 (ICAM), CD106 (VCAM) and HLA-1 as a marker for tissue activation.

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Elevated numbers of CD3-positive lymphocytes (over 10 cells per mm^2) define intramyocardial inflammation. Today, two novel immunohistochemical markers are of increasing relevance for prediction of the clinical course of patients with dilated cardiomyopathy. Perforin-positive, cytotoxic T-cells are detected in biopsies during the acute and chronic phases of infection. An increased number of perforin-positive cells in the initial biopsy is associated with more lesions of cardiomyocytes, poor clinical prognosis of affected patients and increased mortality in a long-term follow-up.

CD45RO is a marker for memory T-cell or activated lymphocytes. A high number of CD45RO-positive cells in the myocardium predicts poor outcome of these patients in a 10-year follow-up. Whereas the absolute number of cellular infiltrates (lymphocytes, macrophages) varies over the course of a disease and might differ from biopsy to biopsy, the expression of adhesion molecules is a global indicator for tissue activation. Introduction of HLA-1 and CD54 in routine biopsy diagnostics improves prediction of inflammatory processes in myocardium without on-site identification of infiltrative cellular foci. Based on immunohistochemical analysis, patients with detectable intramyocardial inflammation without myocytolysis in histology are categorized as borderline myocarditis. Inflammatory processes were diagnosed in more than 40% of all biopated patients, with high predictive value for the clinical course of these patients.

Novel diagnostic markers for cardiomyopathies are microvessel density (MVD) and the quantitative evaluation of fibrosis. In endomyocardial biopsies, MVD is a critical marker in the development of heart muscle diseases. Diagnostics of MVD is performed by the immunohistochemistry of the endothelial surface marker CD31. Chronic inflammation or PVB19 infections of the myocardium reduce MVD, resulting in malfunction of the myocardium and inducing atrophy or hypertrophy of cardiomyocytes. Myocardial fibrosis detected by computer magnetic resonance tomography (CMR) is an independent and incremental predictor of mortality and sudden cardiac death in DCM patients. In biopsies, the quantitative measurement of intramyocardial fibrosis is performed by immunohistochemistry with antibodies specific for Collagen 1 and 3, or by staining with Sirius Red and subsequent digital imaging analysis.

8. Immunohistochemical typing of amyloid deposits

Amyloidosis is a rare but devastating condition caused by deposition of misfolded proteins as aggregates in the extracellular tissues of the body, leading to impairment of organ function. Approximately 25 proteins are recognised to cause amyloidosis and the amyloidogenic protein is the basis for the current classification, however, only four amyloidosis forms (ATTR, AL lambda und kappa, AA) are found mainly in the heart muscle tissue (Tab.1). Each type of amyloid has the prefix ‘A’, for amyloid, followed by an abbreviation derived from the name of the protein.

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Table 3 *Amyloid types*

Amyloid type	Other names	Protein	Mechanism	Associated disease/population
AA	Serum amyloid A	apoSAA	Normal	Sustained, chronic inflammation, Muckie-Wells' Syndrome
AL	Primary (no longer favoured terminology)	Monoclonal Ig light chains (lambda and kappa)	Acquired, abnormal, amyloid-forming protein	Plasma cell dyscrasia/older people
ATTR	Senile systemic (cardiac) amyloidosis	Transthyretin (wild type)	Normal protein, normal concentration, prolonged exposure	Older age (>80), usually male

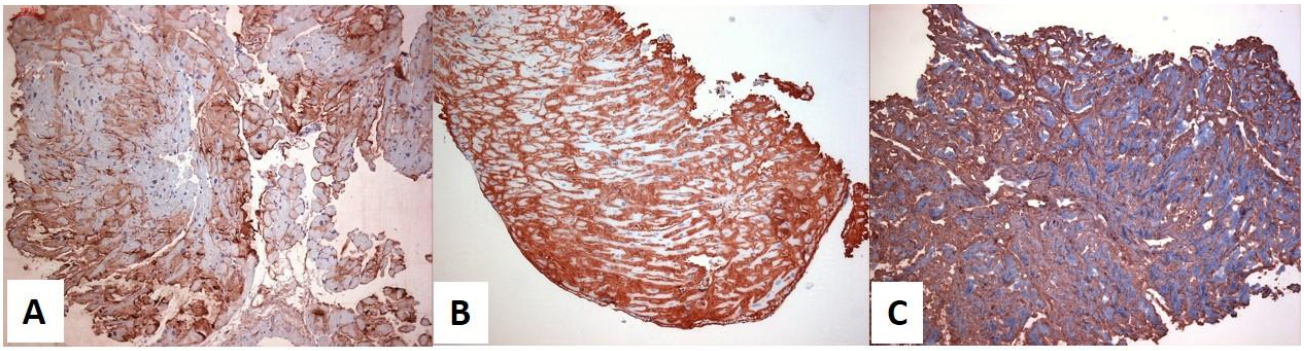

Figure 7 Immunostaining of amyloid deposits with specific antibody and Envision signal amplification: Anti-ATTR (x10) (A), Anti-AL lambda (x10) (B), and Anti-AL kappa (x10) (C).

High clinical suspicion is required to facilitate early diagnosis. Correct identification of the causal amyloid protein is absolutely crucial for clinical management in order to avoid misdiagnosis and inappropriate, potentially harmful treatment, to assess prognosis, and to offer genetic counselling if relevant.

9. Molecular Diagnostics

The molecular diagnostics approach to EMBs is based on detection, quantification and sequencing of viral genomes. With an ever-increasing number of diagnostic tests for virus detection, IKDT focuses on those cardiotropic viruses that have been described as causal triggers of heart failure problems. IKDT's established virus PCR detection methods are listed in Table 4.

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Tests for cardiotropic viruses are based on qualitative detection of virus genomes by nested-PCR and quantification of virus load by quantitative TaqMan PCR. Depending on the two types of viral nucleic acid, we perform the isolation of DNA or RNA in separate extraction procedures. The transcriptional activity of a virus in myocardial tissue or peripheral blood cells is determined for the two most frequent cardiotropic viruses—Erythrovirus and HHV6—by nested-RT-PCR and qPCR.

In order to calculate and standardize estimation of the virus load in small EMBs (viral genomes per µg of isolated human genomic DNA), IKDT applies the most accurate QUANTIFILER TaqMan test (Applied Biosystems, USA). This test was initially developed for forensics to detect minute traces of DNA and is approved by the FDA.

All amplified virus genomes are subjected to sequencing for the determination of existing virus subtypes or infectious variants.

Table 4 *Established tests for cardiotropic viruses in IKDT lab.*

Virus	**Nucleic Acid**	**nested-PCR**	**TaqMan**	**Subtypes/ variants**	**Sequencing of positive PCR**	**Determination of virus subtype by**
Erythrovirus (Parvovirus B19)	DNA RNA	X	X	G1, G2	yes	sequencing
Adenovirus	DNA	X	X	upto 52	yes	sequencing
Human Herpesvirus 6	DNA, RNA	X	X	A and B, ciHHV6	yes	sequencing
Cytomegalovirus	DNA	X		no	yes	
Epstein-Barr-Virus	DNA	X	X	no	yes	
Hepatitis A/B/C	DNA/RNA		X	yes		sequencing
Herpes simplex virus 1 and 2	DNA		X	1 and 2		TaqMan
Coxsackievirus	RNA	X	X	various	yes	sequencing

DNA or RNA is extracted from different endomyocardial biopsies in parallel. During the isolation procedure, each staff member has to care for nuclease-free working conditions (sterile tips, DEPC-treated water, frequent change of gloves and so on). The amount of isolated DNA must be measured by a specialised TaqMan assay. After DNAse digestion, RNA is completely transcribed into cDNA. Finally, both nucleic acid fractions are available as DNA molecules, protected from potential degradation and suitable for long-term storage.

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Detection of viral genomes by nested-PCR

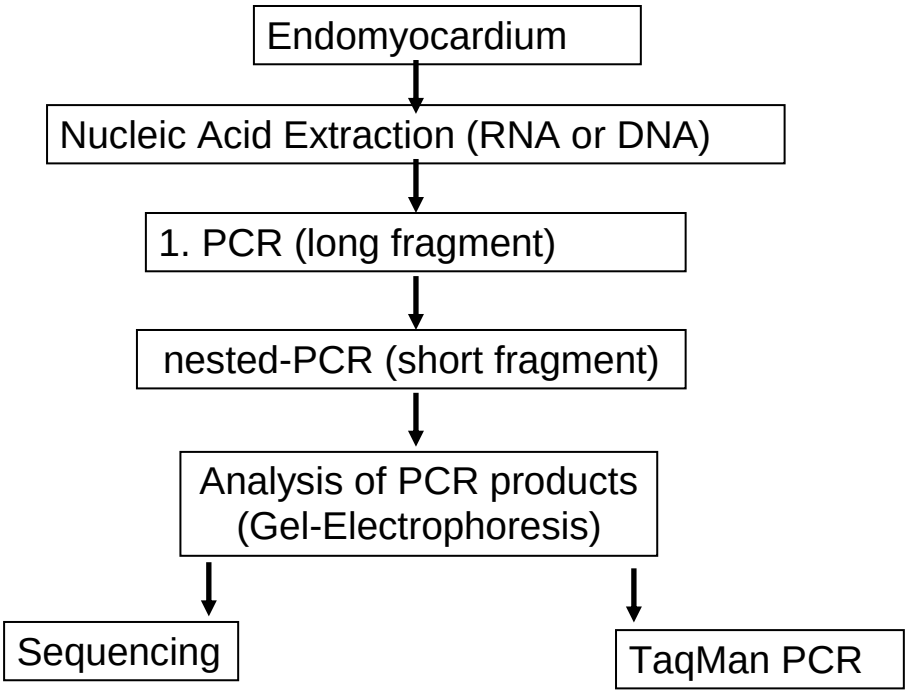

Figure 8 Flowchart of the diagnostic procedure for detection of cardiotropic viruses

We apply nested-PCR for qualitative detection of cardiotropic viruses (Adenovirus (ADV), Coxsackievirus (CVB), Epstein-Barr-Virus (EBV), Erythrovirus (Parvo B19, B19V) and Human Herpesvirus 6 (HHV6)) genome sequences in material extracted from EMB. Nested-PCR is analysed by gel-electrophoresis (Figure 9). For all positive samples, the viral load and the virus genotype will be determined.

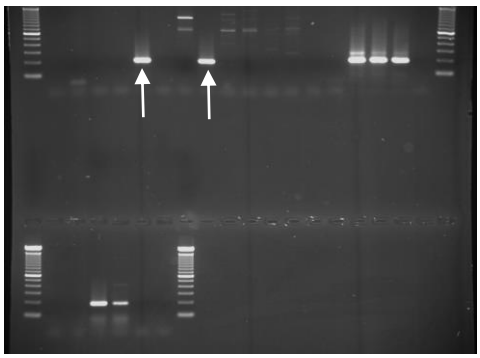

Figure 9 Gel electrophoresis of Coxsackievirus-nested-PCR (*arrows—infected patients*)

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Sequencing of viral genomes

All positive PCR reactions will be sequenced for quality control of preceding nested-PCR and for detection of the amplified virus subtype. We apply double-strand sequencing and subsequent manual alignment against in-house reference files and the NCBI international database (Figure 10). The generated sequences are compared by manual alignment with PHYDE software (Institute of Botanics, Bonn/Dresden) and online with the NCBI database for confirmation of the corresponding virus strain and/or estimation of specific virus subtypes or variants (Figure 10).

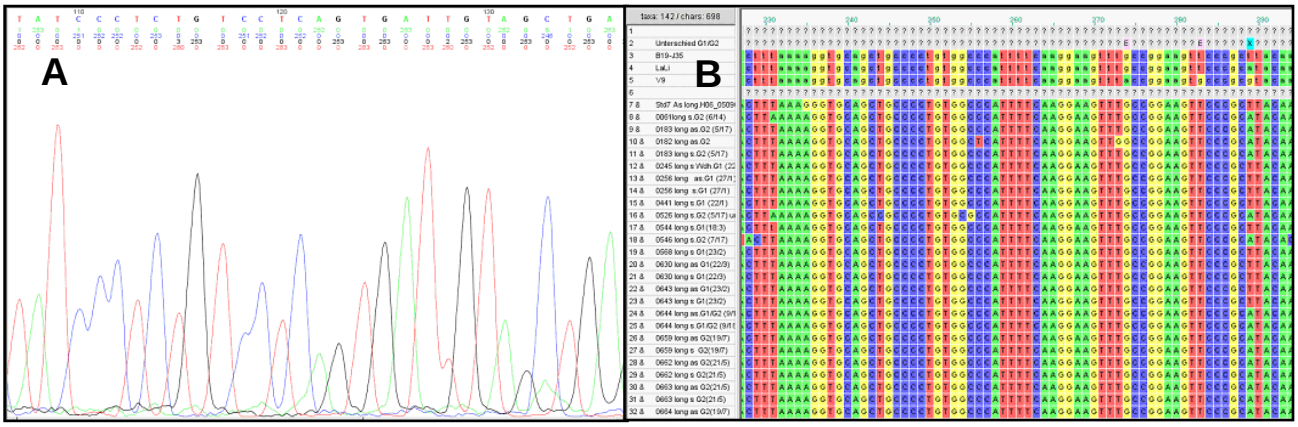

Figure 10 Sequence fragment of Coxsackievirus (A) and alignment of PVB19 genotypes (B)

Quantification of viral load

Successful treatment or therapy of infected patients must be monitored by estimation of the viral load in EMBs at different time points. The viral load in EMB or cells is the ratio of viral genome copies to the associated amount of extracted myocardial tissue, measured by quantification of a housekeeping gene. At IKDT, human genomic DNA content (as an equivalent of extracted biopsy tissue or blood cells) is measured by quantitative QUANTIFILER TaqMan assay, which is recommended for analysis of DNA amount in forensic traces by the US FDA.

Quantitative determination of viral genomes by real-time PCR is based on the additional use of a fluorescent probe in a PCR assay. By simultaneous measurement of a calibration curve, the number of viral gene copies is detected during the amplification process (Figure 11). This extremely sensitive and highly optimized method is unique for estimation of viral loads for DNA viruses in human tissues.

Data for qPCR and sequencing are generated by automated software. Following GLP/GCP guidelines, all raw data and exported files are retained on CD or streamer cassette for long-term storage.

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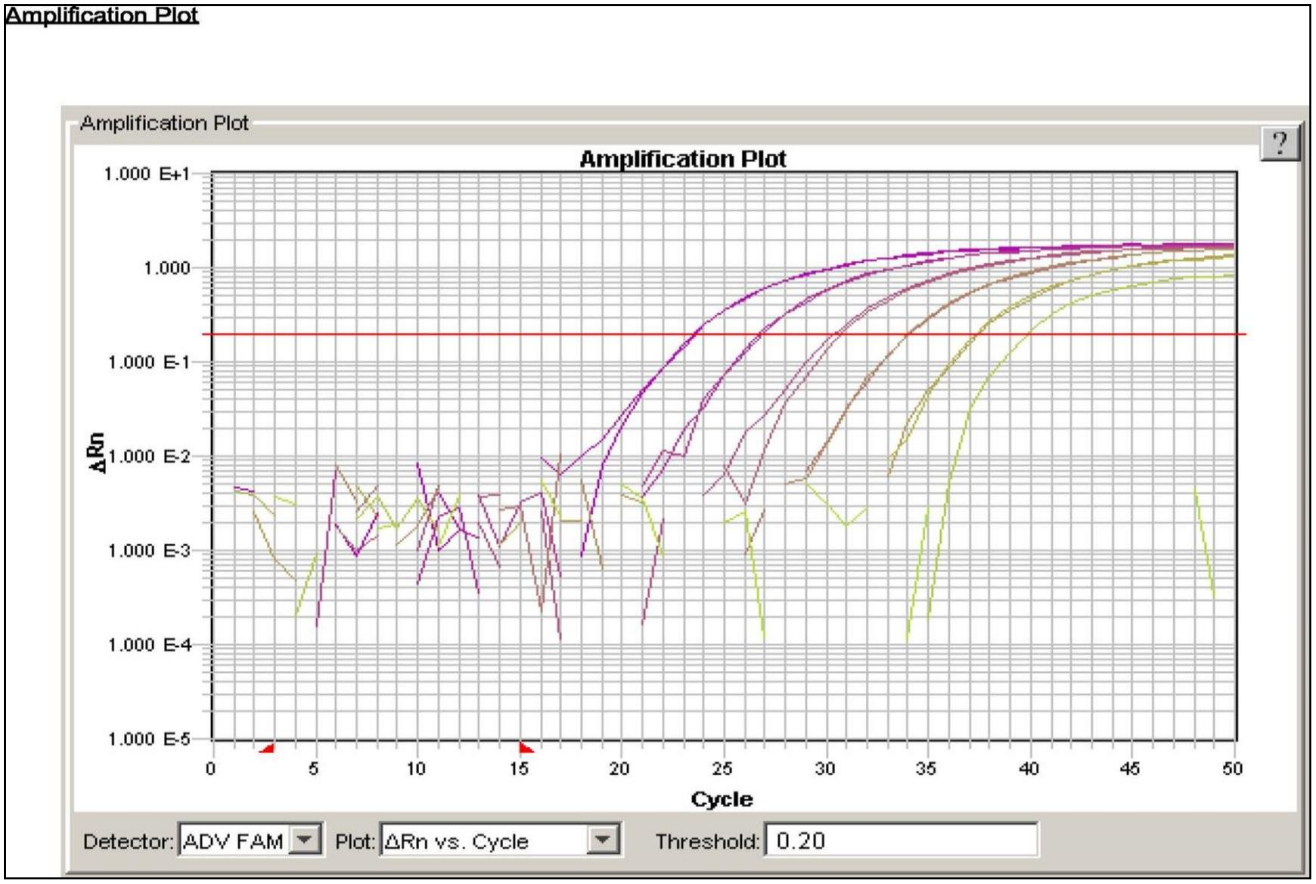

Figure 11 Standard curve for HHV6 TaqMan-qPCR for quantification of viral genomes

Viral transcripts as markers for active replication

The detection of viral genomes or of high viral load in EMB (Erythrovirus, ciHHV-6) may not be predictive for the clinical course of the examined patient. In contrast, the presence of viral RNA transcripts is a sign of viral activity and is more suitable for primary diagnosis and monitoring of the applied therapy.

Monitoring of active replication of Erythrovirus by gene profiling technologies

In 15%–20% of Erythrovirus positive patients, an active virus replication is detectable by measurement of viral RNA, deregulated gene profiles or specific microRNA pattern (Figures 12 and 13). Transcriptionally active erythrovirus is associated with a higher rate of angina, fatigue syndrome or dyspnea. Clinical symptoms of these patients often improve shortly after antiviral treatment with virus replication inhibitors. This process could be monitored by reversibility of the distinctive cardiac expression patterns.

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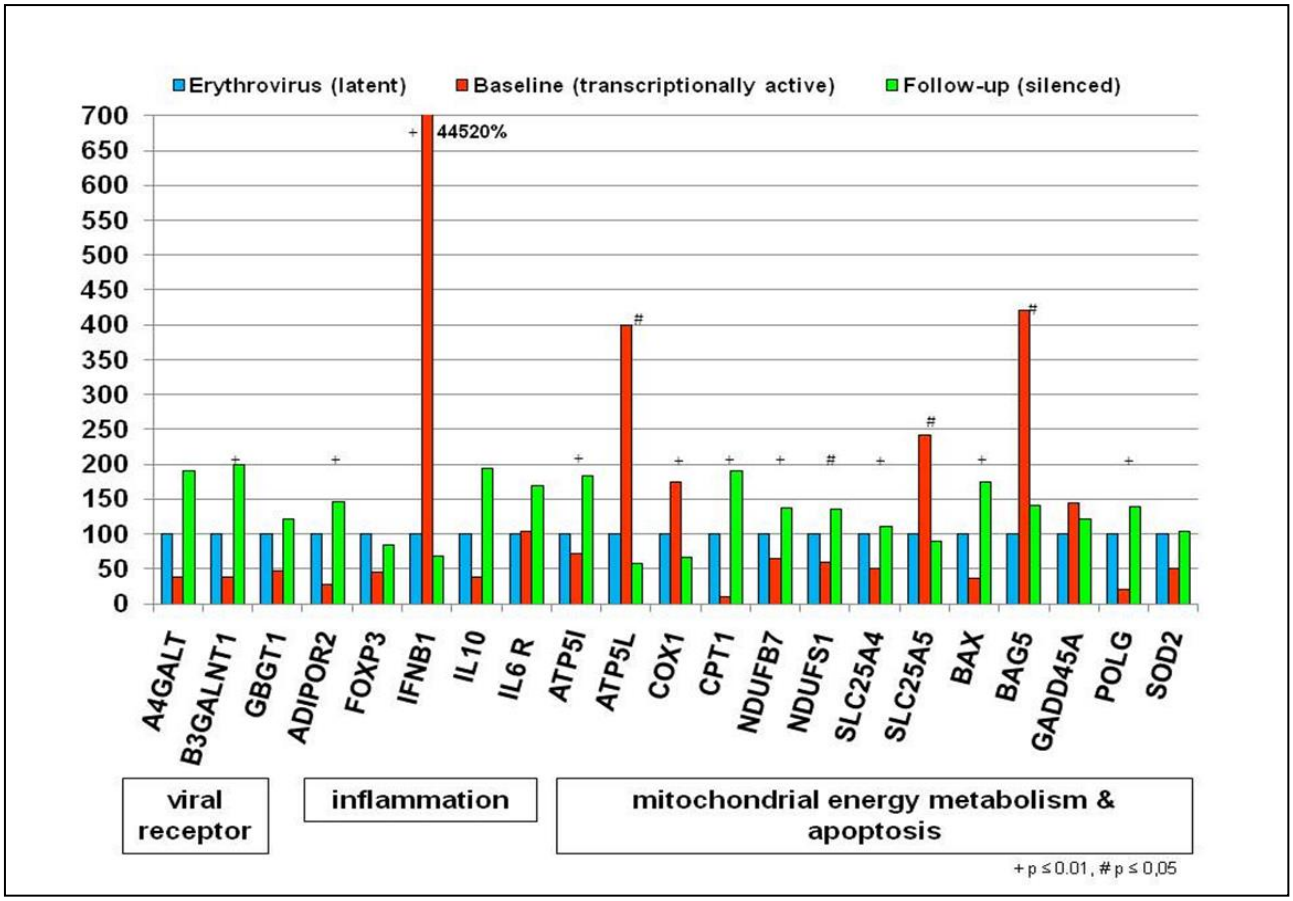

Figure 12 Reversibility of the distinctive cardiac expression patterns associated with functionally active Erythrovirus infection

Gene profiling for diagnosis and therapy monitoring of myocarditis harbouring giant cells

Cardiac inflammatory processes involving giant cells comprise a diverse group of disorders. The distinction between idiopathic giant cell myocarditis (IGCM), a life-threatening and rapidly progressive disease with fatal prognosis, and cardiac sarcoidosis (CS) remains particularly challenging and is frequently delayed until autopsy. IGCM is regarded as a distinct clinical and pathological entity with exclusive cardiac manifestation and poor survival. In contrast, CS is a predominantly systemic disorder with less rapid progression of heart failure but nevertheless fatal outcome in patients with untreated heart involvement. CS is essentially a non-necrotizing process with gradual progression, whereas IGCM is associated with acute myocyte necrosis and extensive early scar formation. Currently, IGCM and CS are diagnosed on the basis of differential patterns of inflammatory cell infiltration and non-caseating granulomas in histological sections of EMB, after heart explantation or postmortem (Figure 13A).

We have established a new method for improved differential diagnosis of IGCM and CS, performing myocardial gene expression profiling in EMBs of these two frequently fatal human myocardial diseases. Myocardial expression profiling using a set of 10–15 altered genes is suitable

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for differentiation of myocarditis harbouring giant cells from active lymphocytic myocarditis (MCA) and facilitates discrimination between IGCM and CS (Figure 13B).
Myocardial gene expression profiling can predict the presence of multinuclear giant cells in the myocardium of patients with clinical suspicion of CS or IGCM, even without direct histological proof from single small EMB sections, so reducing the risk of sampling errors.

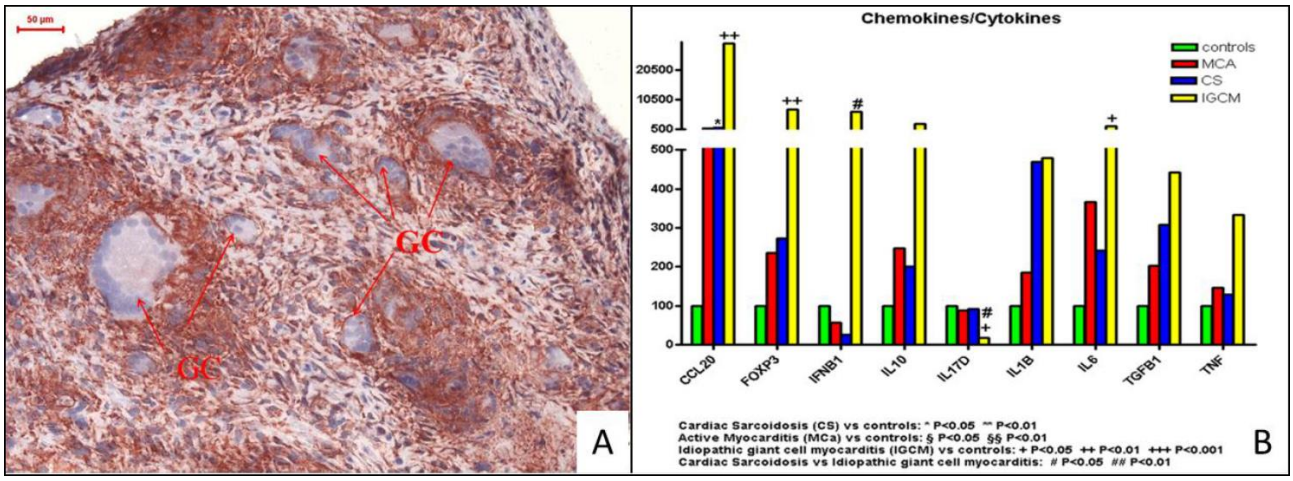

Figure 13 (A) Immunohistological staining of granulomateous myocarditis with CD 11a (LFA-1, T-lymphocytes). Brown staining: Activated inflammatory cells, negative (blue): multinucleated giant cells (GC). (B) Giant cells could be detected by gene profile of highly deregulated genes without pathohistological proof of giant cells. Lifesaving therapy could be initiated by this molecular diagnostics.

Summary

IKDT offers the most comprehensive state-of-the-art facilities and service for analysis of myocardial tissue (EMBs) for morphological abnormalities, viral infections and inflammatory processes as causative factors in cardiomyopathy, with ongoing expansion of protocols to encompass new methods and biomarkers that include microRNA and gene profiles. IKDT provides diagnostic parameters of the examined patient samples and facilitates CRO in clinical trials and research projects, efficiently, reliably and to the highest international standards.

Ganna Aleshcheva, PhD *-Laboratory Director-*
Prof. Ulrich M. Gross, MD *-Medical Director-*
Prof. Heinz-Peter Schultheiss, MD *-CEO-*

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