JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

The prevalence of enteroviral RNA and protein in mitral valves of chronic rheumatic heart disease

Saligrama R Kalpana[1], Regidi Saikant[2], Manjunath C Nanjappa[3], Anita Mahadevan[4], Rudrapatna S Jayshree[2]

[1] *Department of Pathology, Sri Jayadeva Institute of Cardiovascular Sciences and Research, Bangalore, India*
[2] *Department of Microbiology, Kidwai Cancer Institute, Bangalore, India*
[3] *Department of Cardiology, Sri Jayadeva Institute of Cardiovascular Sciences and Research, Bangalore, India*
[4] *Department of Neuropathology, National Institute of Mental Health and Neuro Sciences, Bangalore, India*

Abstract
Introduction: Acute Rheumatic Fever/ Rheumatic Heart Disease (ARF/RHD), a sequel of group A streptococcal (GAS) infection, even today constitutes a public health issue in developing countries including India. Differences in the prevalence of ARF/RHD in countries with a similar prevalence of GAS infections indicate the role of other cofactors in pathogenesis of RHD.
Methodology: We investigated the prevalence of enterovirus (EV) in RHD by probing for both EV RNA and VP1 protein using *Nonisotopic In Situ Hybridization* (NISH) and Immunohistochemistry (IHC) respectively in 75 valvectomy specimens obtained from RHD cases.
Results: Twenty-eight (37%) of the valves showed tissue inflammation with lymphocytic infiltration in a majority of the cases. Twenty-six and 27 (38% and 40%) of the 68 valves showed the presence of EV by IHC and NISH respectively, indicating a very good association between the two tests; however, only about 46 to 48% of them exhibited tissue inflammation. In eight cases (12%) the EV genome was detectable in absence of VP1 protein perhaps indicating a latent viral infection.
Conclusions: Due to a high degree of endemicity of EV in India, we are tempted to speculate that EV may be responsible for the severity and rapid progression of RHD. The virus could either be working synergistically with GAS or could be an opportunist infecting damaged valves.

Key words: Enterovirus; NISH; RHD; mitral valve; Coxsackie virus; IHC.

J Infect Dev Ctries 2021; 15(4):566-572. doi:10.3855/jidc.12503

(Received 01 February 2020 – Accepted 27 May 2020)

Copyright © 2021 Kalpana *et al*. This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction
Acute Rheumatic Fever/ Rheumatic Heart Disease (ARF/RHD) is an autoimmune, multiorgan inflammatory disease, secondary to group A streptococcal (GAS) infection in genetically susceptible individuals [1]. Although RHD has long receded in the developed world, the threat of the disease in developing countries including India is still high [1,2]. Conservative estimates indicate that, of the ~34 million RHD affected individuals and 10 million disability-adjusted life years (DALYs) lost per year [3], 13.2 million live in India [4]. Likewise, of the 1.4 million premature deaths annually worldwide due to RHD [5], nearly 120,000 are estimated to have occurred in India [4]. The global burden of ARF and RHD in low-resource settings is 444/100,000 population, compared with 3.4/100,000 population in non-endemic countries [4,5].

Epidemiological evidence supporting the association between GAS pharyngitis and ARF is strong [5], and RHD development or progression has been prevented with primary and secondary prophylaxis against GAS [3,6-9]. However, a preceding GAS pharyngitis is not an absolute necessity for ARF and likewise, ARF and RHD do not necessarily accompany each other [6-8,10]. Further, there is a striking difference in prevalence of RHD in countries across the world despite similar prevalence of GAS pharyngitis. The low 'hit rate' of RHD following GAS pharyngitis (0.05% of >600 million) [2,10,11] points towards the role of additional cofactors in the pathogenesis of RHD. Co-infections with other viral pathogens – e.g. Coxsackie B, is a strong possibility as seen in both ARF and RHD [12-14]. Considering that EV is endemic in India and that ARF and chronic RHD too are significant problems, we thought it would be pertinent to investigate the prevalence of EV RNA and VP1 protein in rheumatic mitral valves using *Nonisotopic In Situ Hybridization* (NISH) and Immunohistochemistry (IHC) respectively.

Methodology

Ethics Statement

The study was approved by Institutional Medical Ethics Committee of Sri Jayadeva Institute of Cardiovascular Sciences and Research (SJICR) (vide no.SJIC/RES/23dt.24thSeptember,2010). Written informed consent for using the samples for research purposes was obtained from all the patients/guardians prior to surgery.

Study Group

Seventy-five patients with chronic RHD (36 males and 39 females; mean age 38.06 ± 11.5 years) scheduled to undergo mitral valve replacement surgery, between the years 2010 and 2017at Sri Jayadeva Institute of Cardiovascular Sciences and Research (SJICR), Bangalore, a tertiary cardiac care center comprised the study group.

Controls

Thirty age matched individuals (28 males and 2 females; mean age 34.77 ± 13.52 years) who succumbed to road traffic accidents (RTA) at the National Institute of Mental Health And Neurosciences (NIMHANS), Bangalore formed the control for mitral valves.

Specimens

Venous blood was collected from all the patients of the study group before surgery. Surgical specimens of mitral valves received in the Department of Pathology, SJICR, formed the study material. Normal mitral valves collected at autopsy, from victims of RTA in Department of Neuropathology, (NIMHANS) served as controls.

Positive control: Coxsackie B2 virus infected mouse tissue (kind gift from Dr. S. A Huber); digoxigenin labeled group specific oligonucleotide probe complementary to the conserved group common sequence in the 5' non coding region of the EV genome (5'to3' GAA ACA CGG ACA CCC AAA GTA GTCGGT TCC GCT GCR GAG TTR CCC RTT ACG ACA) [15-17]; digoxigenin labeled β actin RNA probe (Cat.No.11498045910, Roche Diagnostics GmbH, Mannheim, Germany); Anti DIG antibody (Anti-Digoxigenin-AP Fab fragments, Cat.No.11093274910, Roche Diagnostics GmbH) 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)/Nitro Blue Tetrazolium (NBT) substrate (Sigmafast, Cat.No.B5655, St. Louis, Missouri, USA).

Monoclonal antibody against enteroviral capsid protein VP1 (Clone 5-D81, Dako, Agilent, Santa Clara, CA, USA) – immunogenic towards B5 antigen, which therefore has been shown to react with most of the enterovirus strains of the coxsackie, echo and poliovirus groups; secondary antibody kit (Catalogue No. K5007; Dako REAL EnVision Detection System, Peroxidase/DAB+, Santa Clara, CA, USA). Total leukocyte count (Coulter Ac. T-5 part differential cell counter), C-Reactive Protein (CRP) (Card method, Span Diagnostics), Bangalore, India, Erythrocyte Sedimentation Rate (ESR) (modified Wintergreen method) and Anti Streptolysin O antibodies (ASO) (Rhelax-ASO Tulip Diagnostics, Goa, India.

Nonisotopic In Situ Hybridization

NISH was carried out as per the protocol described earlier [15]. The integrity of RNA in tissue sections was verified by NISH using digoxigenin labeled β actin RNA probe. With every batch of NISH, EV infected mouse tissue sections served as positive controls and no probe tissue sections served as negative controls.

Scoring NISH

A blinded, independent microscopic scoring of the stained slides was carried out by three senior pathologists. Sections were considered to express EV RNA only when the intracytoplasmic signals were consistently localized to the same region in serial sections upon repeat testing. Also, a slide was labeled as positive only when at least two of the three pathologists consensually agreed upon the scoring. All positives were confirmed by a repeat NISH test on a different day.

Hematoxylin and Eosin stained tissue sections of the valve were graded for cellularity and interstitial inflammatory cell infiltrate.

Quantitative reverse transcription PCR was attempted to validate NISH results, insufficient yield of RNA however compelled us to abandon this procedure.

Immunohistochemistry

The presence of EV in the tissues was subsequently validated by indirect immunohistochemistry using antibodies to VPI. EV infected mouse tissue constituted positive control (detailed under NISH) and tissue sections without addition of primary antibodies served as negative controls. Three pathologists (SRK, AM, RSJ) blinded to groups independently scored each slide as positive and negative. Intracytoplasmic staining consistently localized to the same region on serial sections and upon repeat testing was considered positive with at least two of the three pathologists consensually agreeing upon the scoring.

Statistical tests

Chi-square test was used to calculate the association between various parameters.

Results

Demographic and clinical findings

The study cohort included 75 cases (36 males and 39 females) with a mean age of 38.06 ± 11.5 years. CRP was positive in 37% (28/75), while ASO antibodies were detectable in only 4% (3/75) of the cases. ESR was raised in 31% (23/75) and total leukocyte count was increased in 31% (23/75) of patients (Supplementary Table 1).

Histopathological findings

Cellularity of the valves showed a wide variation from 5 to 80%. Interstitial valve inflammation was found in 37.33% (28/75); mononuclear cell infiltration in 22.67% (17/75) and mixed inflammatory cells in

Figure 1. Cellularity of the resected valves from RHD cases varied widely. A representative tissue section of a mitral valve showing fibrosis and neoangiogenesis (40x Hematoxylin and Eosin).

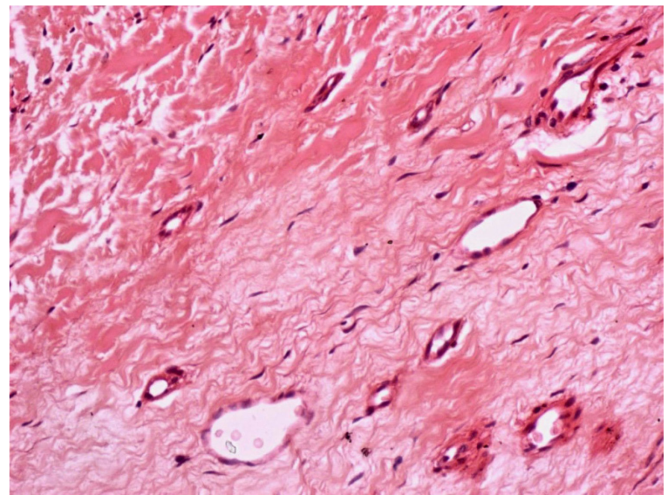


Figure 2. Distribution of EV RNA in mitral valves.

Tissue sections of mitral valves were probed by NISH using digoxigenin labeled group specific EV probe. **A.** A representative tissue section of a case scored as positive showing the distribution of the Enteroviral RNA in the myofibroblasts of the Mitral Valve (10X; Inset 40 X). **B.** FFPE tissue sections from EV infected mouse were probed with the EV probe with every batch of NISH staining and served as positive control. A representative tissue section depicting intense positivity for Enterovirus RNA (10 X). **C.** The integrity of RNA in all the cases was verified by performing a beta actin RNA NISH. A representative picture of the same case as in 2A. is shown showing good integrity of RNA. **D.** Control valves failed to express EV RNA. A representative picture at 40X magnification.

14.67% (11/75) of the cases (Figure 1; Supplementary Table 1).

Prevalence of Enterovirus in mitral valves

The prevalence of the virus could be analyzed for expression of both EVRNA and VP1 protein in 68 of the 75 cases.

NISH

Positive signals were seen within cells morphologically resembling myofibroblasts and endothelial cells and smooth muscle cells of the newly formed vessels in the valve (Figure 2). Enteroviral RNA was detected in 27/68 valves (40%) (Table1).

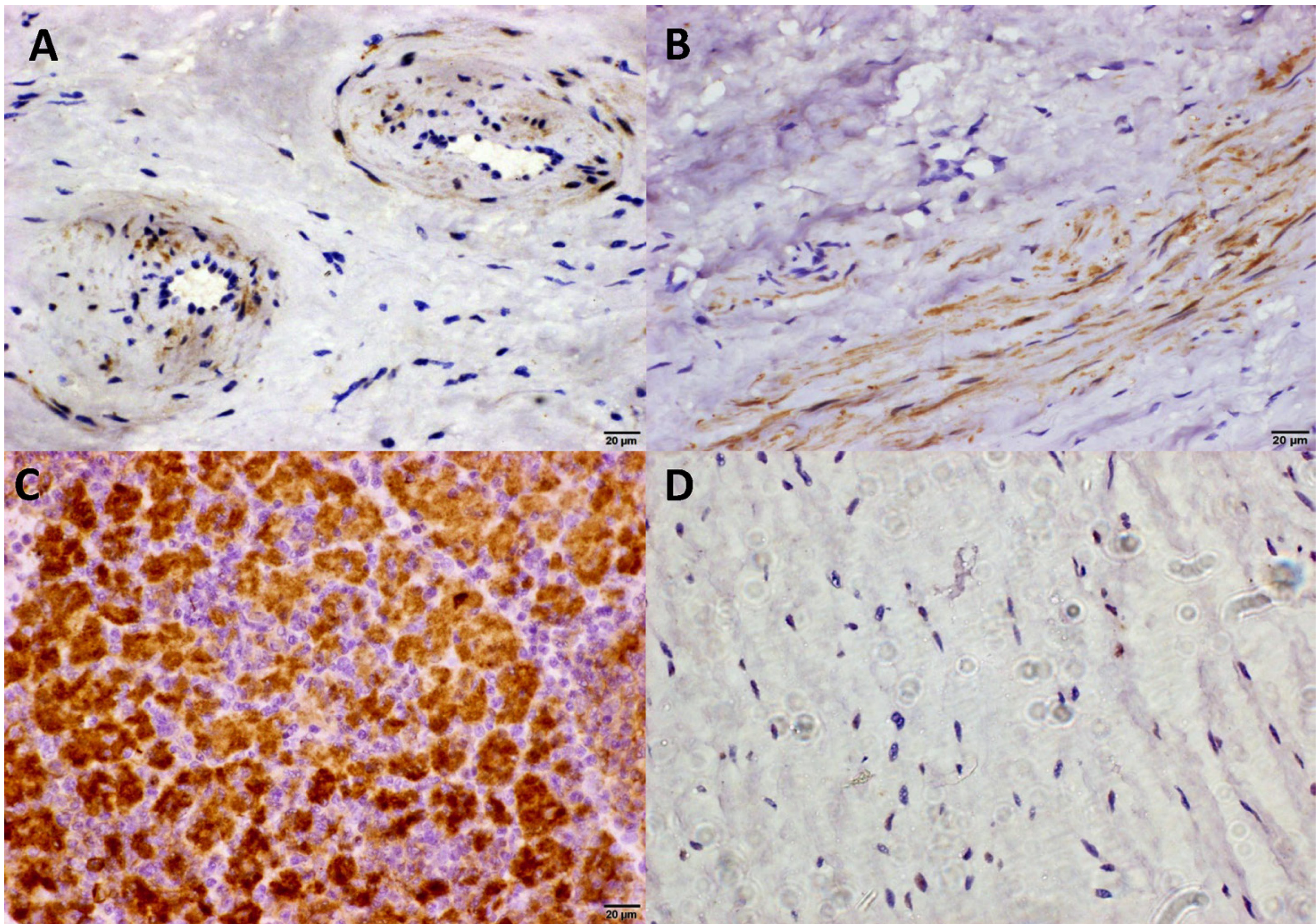
IHC

VP1antigen was detected in 38% of mitral valves (26/68) demonstrating active viral replication (Figure 3).

Hence, out of the 68 cases, 19 (28%) were positive and 34 (50%) negative, by both NISH and IHC, thereby exhibiting a concordance between the tests. Eight cases (8/68, 12%) were positive by NISH, but negative by IHC that may represent latent / dormant viral infection (Table 1) and likewise seven cases (7/68, 10%) positive by IHC, were negative by NISH probably secondary to viral RNA degradation (Table 1), during the procedure.

While there was a significant association between the detection of the viral RNA and protein ($p<0.0001$; Table 1), there was no association between tissue inflammation and prevalence of the virus either by NISH or IHC ($p>0.5$; Tables 2 and 3). No viral

Figure 3. Expression of EV protein in mitral valves.



FFPE sections of valves were stained for VP1 capsid protein of EV by IHC. **A.** A representative section showing EV capsid protein in the vascular smooth muscle cells of the Mitral Valve (10X). B Positive signals in the myofibroblasts from an adjacent field. **C**. FFPE tissue sections from EV infected mouse were stained using anti VP1 antibodies as the primary and with every batch of IHC staining and served as positive control. A representative tissue section depicting intense positivity for Enterovirus Capsid protein (20X). **D.** None of the control valves showed expression of VP1 protein. A representative picture at 40X magnification.

component was detected in control valves either by IHC or NISH (n = 30) (Figure 2D and 3C).

Discussion

Rheumatic Heart Disease is still a major public health concern in developing countries, and India has the dubious distinction of being declared the "RHD capital of the world" [4]. Acute Rheumatic Fever and RHD are known to be post GAS sequel. Various factors influence ASO reactivity in RHD, which could vary from negative to high positive and reinfection with GAS augments sustained or continuous rise in antibody titers [2-3,18].We found only 4% of RHD cases to be ASO reactive; one of the reasons for this could be ascribed to Penicillin prophylaxis.

There remain several unanswered questions in the etiopathogenesis of RHD. Indeed, the classical sequence of GAS pharyngitis, followed by ARF, progressing to RHD does not appear in the majority of RHD: in only ~66% of the patients with ARF a history of pharyngitis could be elicited and similarly, only ~60% of patients with ARF progressed to RHD [6-7]. It is still not clear why only a small fraction of GAS pharyngitis patients worldwide, develop RHD [2,5]. Amongst the various factors contributing to the etiopathogenesis of RHD, co-existing viral infections also feature to play a role [14].Of the many viruses implicated in the etiopathogenesis of RHD, the contribution of Enteroviruses in particular viruses of the Coxsackie group, is thought to be significant [19]. While an Indian study carried out in the 90s recorded Coxsackie B virus antibodies in about 91% of patients with ARF but considered it incidental [13], an Egyptian study found a low association between Coxsackie B viral infection and ARF and chronic RHD [20]. However, direct demonstration of the virus or its proteins in the lesions of RHD reveal the true prevalence in any geographical region [14,21-25]. Replicating forms of the virus have also been shown in cardiac tissues, strengthening the association of the virus with RHD [15]. The latter Chinese study, found that while 24% of the mitral valves harbored viral RNA, almost twice this number expressed viral protein. In the present study, ~40% of the RHD valves showed presence of EV both by NISH and IHC. Though some studies in the past have reported cross reactivity of 5-D8/1 clone of antibodies with heat shock proteins and to uninfected human cardiomyocytes [26,27], we and others didn't observe any positive signals in normal control valves [15]. Valves which showed the presence of the viral genome without expression of viral protein were interpreted to be dormant infections; particularly since viruses are known to remain latent for long periods in the cardiac tissues following systemic spread, and become reactivated into an acute fulminating infection with rapidly progressive myocardial disease

Table 1. Association between NISH and IHC for prevalence of EV in RHD.

		NISH (n)		
		Positive	**Negative**	**Total**
IHC (n)	Positive	19	7	26
	Negative	8	34	42
	Total	27	41	68

There was a significant association between the prevalence of the virus in the tissues by IHC and NISH ($p < 0.0001$).

Table 2. Association between Inflammation and IHC prevalence of EV in the cardiac valves of chronic RHD.

		Inflammation (n)		
		Positive	**Negative**	**Total**
IHC (n)	Positive	12	14	26
	Negative	15	27	42
	Total	27	41	68

There was no association between inflammation and presence of EV in the tissues ($p = 0.45$).

Table 3. Association between Inflammation and NISH prevalence of EV in the cardiac valves of chronic RHD.

		Inflammation (n)		
		Positive	**Negative**	**Total**
NISH (n)	Positive	13	14	27
	Negative	15	33	48
	Total	28	47	75

There was no association between inflammation and presence of EV in the tissues by NISH ($p=0.21$).

and death [28,29]. Although we did not use EV anti-genome as a probe to confirm the findings obtained by NISH, expression of the viral protein VP1 was considered as proof of viral replication in the tissues. Moreover, detecting viral capsid protein is more sensitive than detecting RNA, which is prone to fragmentation and degradation in FFPE tissues [15]. In the present study, only 46% to 48% of EV infected valves exhibited a tissue inflammatory response, representing active viral infection.

The alternative possibility of EV playing an opportunistic role by establishing itself in a damaged RHD milieu cannot be ruled out. However, the chances of the virus playing an active role are higher considering the renewed thinking on the etiopathogenesis of the disease [26]. Molecular mimicry between M protein of GAS and cardiac myosin [27] and between Coxsackie (CX) viral proteins and cardiac actin [28] has been known for some time now. Additionally, the elegant demonstration of molecular complementarity between GAS and CX; cardiac actin and myosin; and laminin and collagen IV has added a plausible dimension to the synergism of GAS and CX in the pathogenesis of RHD [26].

Conclusions

Considering the fact that India is endemic to both the microbes: GAS and EV, the results of the present study lend credence to the possibility that the two organisms could collaborate in the causation of RHD. The synergism between the two microbes may perhaps explain the severity and rapid progression of the disease in this part of the world. Alternatively, the possibility of EV being an opportunist colonizing the damaged valves cannot be entirely ruled out [30].

Acknowledgements

The authors wish to express their gratitude to Dr. S.A. Huber, Department of Pathology, University of Vermont, Vermont, USA for her generous gift of tissue blocks of EV infected mice. We wish to thank Sunil Kumar, Sr. technologist, SJICR, Bangalore and Simran Jain, Junior Research Fellow, Department of Pathology, SJICR, Bangalore for the technical help; Shankar S.K. Professor Emeritus and Principal coordinator Brain Bank, NIMHANS, for the generous help of controls valves; Clementina Rama Rao, Professor, Department of Pathology, and Mahua Sinha, Assistant Professor, Department of Microbiology, Kidwai Cancer Institute for scoring the NISH slides; Alka Murali, Junior Research Fellow, Department of Pathology, SJICR, Bangalore for her help in computing the data, and Dr. Mariamma Phillip, Associate Professor (Biostatistics), NIMHANS, Bangalore for her help in statistical analysis.

Authors' contributions

SRK and RSJ conceived and designed the study, wrote the manuscript, RS did all the bench work, MCN did clinical work up and actively facilitated sample procurement from patients, AM and SRK screened and scored the IHC slides, SRK also screened the ISH slides.

Funding

This work was supported by funds from Rajiv Gandhi University of Health Sciences to SRK (RGUHS/R&D/Researchgrants/M23/2010-11dt25.11.2010); Sri Jayadeva Institute of Cardiovascular Sciences and Research; and Roche (dt 11.2.2011).

Statement of Ethics

The research was conducted ethically in accordance with the Helsinki. Subjects (or their parents or guardians) gave their written informed consent and the study was approved by the Institutional Ethics Committee of the Institute.

References

1. Watkins DA, Johnson CO, Colquhoun SM, Karthikeyan G, Beaton A, Bukhman G, Forouzanfar MH, Longenecker CT, Mayosi BM, Mensah GA, Nascimento BR, Ribeiro ALP, Sable CA, Steer AC, Naghavi M, Mokdad AH, Murray CJL, Vos T, Carapetis JR, Roth GA (2017) Global, regional, and national burden of rheumatic heart disease, 1990-2015. N Engl J Med 377:713-722.
2. Brahmadathan NK (2017) Molecular biology of group a Streptococcus and its implications in vaccine strategies. Indian J Med Microbiol 35: 176-183.
3. Sanyahumbi AS, Colquhoun S, Wyber R, Carapetis JR (2016) Global disease burden of group A Streptococcus. In: Ferretti JJ, Stevens DL, Fischetti VA, editors. Streptococcus pyogenes Basic Biology to Clinical Manifestations. Oklahoma City, USA: University of Oklahoma Health Sciences Center. 661-704.
4. Karthikeyan G (2017) Rheumatic heart disease in India: Declining, but not fast enough. Natl Med J India 30: 247-248.
5. Zühlke L, Engel M E, Karthikeyan G, Rangarajan S, Mackie P, Cupido B Mauff K, Islam S, Joachim A, Daniels R, Francis V, Ogendo S, Gitura B, Mondo C, Okello E, Lwabi P, Al-Kebsi MM, Hamman CH, Sheta SS, Haileamlak A, Daniel W, Goshu DY, Abdissa SG, Desta AG, Shasho BA, Begna DM, ElSayed A, Ibrahim AS, Musuku J, Thomas FB, Okeahialam BN, Ige O, Sutton C, Misra R, Fadl AA, Kennedy N, Damasceno A, Sani M, Ogah OS, Olunuga T, Elhassan HHM, Mocumbi AO, Adeoye AM, Mntla P, Ojji D, Mucumbitsi J, Teo K, Yusuf S, Mayosi BM (2015) Characteristics, complications, and gaps in evidence-based interventions in rheumatic heart disease: the Global Rheumatic Heart Disease Registry (the REMEDY study). Eur Heart J 36: 1115–1122.
6. Carapetis JR, Zühlke L, Taubert K, Narula J (2013) Continued challenge of rheumatic heart disease: the gap of understanding or the gap of implementation? Glob Heart 8: 185–186.

7. Carapetis JR, Steer AC, Mulholland EK, Weber M (2005) The global burden of group A streptococcal diseases. Lancet Infect Dis 5: 685–694.
8. Sika-Paotonu D, Beaton A, Raghu A, Steer A, Carapetis J (2016) Acute Rheumatic Fever and Rheumatic Heart Disease. In: Ferretti JJ, Stevens DL, Fischetti VA, editors. *Streptococcus pyogenes* Basic Biology to Clinical Manifestations. Oklahoma City (OK): University of Oklahoma Health Sciences Center. 771-826.
9. Lennon D, Kerdemelidis M, Arroll B (2009) Meta-analysis of trials of streptococcal throat treatment programs to prevent rheumatic fever. Pediatr Infect Dis J 28: e259–264.
10. Dougherty S, Beaton A, Nascimento BR, Zühlke LJ, Khorsandi M, Wilson N (2018) Prevention and control of rheumatic heart disease: Overcoming core challenges in resource – poor environments. Ann Pediatr Cardiol 11: 68-78.
11. Bright PD, Mayosi BM, Martin WJ (2016) An immunological perspective on rheumatic heart disease pathogenesis: more questions than answers. Heart 102: 1527-1532.
12. Root-Bernstein R, Vonck J, Podufaly A (2009) Antigenic complementarity between coxsackie virus and streptococcus in the induction of rheumatic heart disease and autoimmune myocarditis. Autoimmunity 42: 1-16.
13. Suresh L, Chandrasekar S, Rao RS, Ravi V, Badrinath S (1989) Coxsackie virus and rheumatic fever. A correlative study. J Assoc Physicians India 37: 582-585.
14. Li YW, Yang YZ, Chen HZ (1997) Relationship between enteroviral infection and chronic rheumatic valvular disease. Chin J Exper Surg 14: 163–164.
15. Li Y, Pan Z, Ji Y, Peng T Archard LC, Zhang H (2002) Enterovirus replication in valvular tissue from patients with chronic rheumatic heart disease. Eur Heart J 23: 567-573.
16. James Howard Pringle (1995) Non-isotopic detection of RNA in situ. In: Levy ER, Herrington CS, editors. Non-isotopic methods in molecular Biology, A Practical Approach. 1stEdition, Oxford, USA: Oxford University Press. 85-110.
17. Oikarinen M, Tauriainen S, Penttilä P, Keim J, Rantala I, Honkanen T, Hyöty H (2010) Evaluation of immunohistochemistry and in situ hybridization methods for the detection of enteroviruses using infected cell culture samples. J Clin Virol 47: 224-228.
18. Kotby AA, Habeeb NM, Ezz El Elarab S (2012) Antistreptolysin O titer in health and disease: levels and significance. Pediatr Rep4: e8.
19. Muir P (2002) Enterovirus infection of the heart a causal or contributory factor in chronic rheumatic heart disease? Eur Heart J 23: 517-520.
20. Donoso Mantke O, Meyer R, Prösch S, Niedrig M (2004) Frequent detection of viral nucleic acids in heart valve tissue. J Clin Microbiol 42: 2298-2300.
21. Zaher SR, Kassem AS, Hughes JJ (1993) Coxsackie virus infections in rheumatic fever. Indian J Pediatr 60: 289-298.
22. Burch GE, Sun SC, Chu KC, Sohal RS, Depasquale NP (1967) Coxsackie B viral myocarditis and valvulitis identified in routine autopsy specimens by immunofluorescent techniques. Am Heart J 74: 13–23.
23. Burch GE, Harb JM, Hiramoto Y (1974) Human valvular disease in Coxsackie virus B4 infection: a light immuno-fluorescent and electron microscopic study. Cardiology 59: 83–91.
24. Pongpanich B, Boonpucknavig S, Wasi C, Tanphaichitr P, Boonpucknavig V (1983) Immunopathology of acute rheumatic fever and rheumatic heart disease. The demonstration of Coxsackie group B viral antigen in the myocardium. Clin Rheumatol 2: 217–222.
25. Ward C, Ward AM (1974) Viral antigen in valvular heart disease. Lancet 1: 755–756.
26. Schoub BD, Johnson S, McAnerney JM, Dos Santos IL, Klaassen KI (1985) Epidemic Coxsackie B virus infection in Johannesburg, South Africa. J Hyg 95: 447–55.
27. Root-Bernstein R (2014) Rethinking molecular mimicry in rheumatic heart disease and autoimmune myocarditis: Laminin, collagen IV, CAR, and B1AR as initial targets of disease. Front Pediatr 2: 85.
28. Cunningham MW, Antone SM, Gulizia JM, McManus BM, Fischetti VA, Gauntt CJ (1992) Cytotoxic and viral neutralizing antibodies cross react with streptococcal M protein, enteroviruses, and human cardiac myosin. Proc Natl Acad Sci USA 89: 1320-1324.
29. Tay L, Yin-Murphy M, Chua PH, Koh LH (1983) Prevalence of coxsackie virus B antibody in patients with suspected rheumatic fever and rheumatic heart disease. Singapore Med J 24: 37-40.
30. Andréoletti L, Bourlet T, Moukassa D, Rey L, Hot D, Li Y, Lambert V, Gosselin B, Mosnier JF, Stankowiak C, Wattré P (2000) Enteroviruses can persist with or without active viral replication in cardiac tissue of patients with end-stage ischemic or dilated cardiomyopathy. J Infect Dis 182: 1222-1227.

Corresponding author
Dr. S. R. Kalpana,
Professor and Head, Department of Pathology,
Sri Jayadeva Institute of Cardiovascular Sciences and Research,
Bannerghatta Main Road, Jayanagar 9th Block, Bangalore,
Karnataka, India.
Tel: 91-80-22977305
Fax: 91-80-26534477
Email: kalpanasr@gmail.com

Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Table1. Master chart of clinical and laboratory data.

Slide No.	Hosp. No.	Age	Sex	Diagnosis	ASO	CRP	TC	ESR mm/hr	Cellularity (%)	Inflammation	EV RNA NISH	IHC
1	264323	53	F	RHD, moderate MS with MR, severe TR	Negative	Negative	6,700	20	20	Absent	Negative	Negative
2	264341	30	F	MS	Negative	Negative	5,000	12	50	Absent	Negative	Negative
3	264371	25	F	RHD, severe MS, moderate MR, PH severe AR, AF	Negative	Positive	14,000	0.5	15	Absent	Negative	Negative
4	244334	38	F	Severe MR with AR	Negative	Negative	12,700	44	5	Absent	Negative	Negative
5	264869	62	M	Severe MR with PH, AF	Negative	Negative	5,800	2	40	Absent	Negative	Negative
6	264372	42	F	RHD, severe MS, moderate MR, with PH, severe AR	Negative	Positive	14,900	0.5	5	Absent	Negative	Positive
7	266311	48	M	Severe MS, mild MR, moderate MR, PH, AF, Mild AR	Negative	Negative	8,200	0.2	20	Absent	Negative	Negative
8	266948	40	M	MS	Negative	Negative	8,700	0.5	20	Absent	Positive	Positive
9	267392	24	M	Severe MS and moderate MR	Negative	Positive	22,600	34	60	Absent	Negative	Not done
10	268733	40	F	Severe MS, Mild AR	Negative	Negative	10,200	30	50	√ L	Positive	Positive
11	277859	49	M	MR	Negative	Negative	7,800	10	30	Absent	Negative	Negative
12	274858	45	M	RHD, severe MS, moderate MR, AF, severe TR with PH	Negative	Negative	6,900	14	60	Absent	Negative	Not done
13	275793	45	F	RHD MS, moderate MR	Negative	Negative	10,100	26	30	Absent	Negative	Negative
14	276416	49	F	RHD, severe MS, PH, AF	Negative	Negative	6,100	08	40	Absent	Positive	Negative
15	275393	35	F	RHD, moderate MS, severe MR, moderate MS, mild AR, mild TR, mild PH, AF	Negative	Negative	9,000	0.5	30	√ L	Negative	Not done
16	276418	25	M	RHD, severe MS, mild MR, mild AR, mild TR, PH AF	Negative	Negative	8,800	12	70	Absent	Negative	Negative
17	275738	37	F	MS, MR	Negative	Negative	6,000	08	60	√ L	Negative	Negative
18	277104	34	F	RHD, moderate MR, TR	Negative	Positive	9,000	40	50	√ L	Negative	Negative
19	276719	40	F	Moderate MR with MS	Negative	Positive	8,400	10	50	Absent	Negative	Negative
20	279144	44	F	RHD, severe MS, trivial MR, moderate TR, PH	Negative	Positive	8,900	26	60	Absent	Negative	Negative
21	278342	60	M	RHD, severe MS, moderate TR, AF	Negative	Negative	5,700	12	70	√ L+N infected vegetation	Negative	Negative
22	279868	40	F	RHD, severe MR, TR AF	Negative	Negative	6,300	0.6	20	Absent	Negative	Negative
23	275547	30	F	RHD, severe MR, severe TR, moderate MS	Negative	Positive	10,400	18	30	Absent	Negative	Negative
24	286984	25	F	RHD, severe MR	Negative	Negative	7,900	10	40	Absent	Negative	Negative
25	287084	33	F	RHD, severe MR, trivial TR, PH	Negative	Positive	10,000	10	50	Absent	Positive	Positive
26	288145	59	F	RHD, severe MS, MR, severe PH, trivial AR, mild TR	Negative	Positive	5600	48	20	Absent	Negative	Negative
27	287951	34	M	RHD, severe MS, severe TR, AF	Negative	Positive	11,000	0.8	20	Absent	Negative	Negative
28	287999	55	M	RHD, moderate MS, MR	Negative	Positive	8,600	0.8	40	√ L	Positive	Positive
29	285396	45	F	Moderate MS ,MR, severe TR	Negative	Positive	11,600	20	5	Absent	Positive	Positive
30	281753	36	M	RHD, severe AR, moderate MR	Negative	Positive	12,500	30	70	√ L+P	Negative	Negative
31	282364	50	F	RHD, severe MS, severe TR, AF	Positive	Positive	4,700	44	30	Absent	Negative	Positive
32	283876	25	M	RHD, severe MS	Negative	Positive	7,600	20	20	Absent	Negative	Not done
33	284565	19	M	Severe AR, MR, moderate TR	Positive	Positive	7,300	14	60	Absent	Positive	Positive
34	233886	19	M	Severe MS, moderate MR, moderate AR, mild AS	Negative	Negative	17,000	08	40	√ L	Negative	Negative
35	234014	38	F	Moderate MS, severe MR, mild TR, PH	Negative	Negative	7,500	32	50	√ L	Positive	Positive
36	233523	30	M	Calcific MS, mild MR,AR	Negative	Negative	6,100	14	20	√ L	Negative	Positive
37	101242	40	M	Severe AS, moderate MS, mild MR, mild AR	Negative	Positive	6,900	12	20	√ L+N	Positive	Negative
38	102838	28	F	RHD, severe MR, mild AR, moderate TR	Negative	Positive	8,600	18	40	√ L	Negative	Negative
39	234988	48	F	RHD, severe MS	Negative	Positive	16,200	92	20	√ L	Positive	Positive
40	235470	18	M	RHD, severe MR, moderate AR	Negative	Negative	10,100	05	70	√ N+L	Positive	Positive
41	235741	22	M	severe MR, moderate AR, mild TR, PH	Negative	Negative	7,200	07	80	√ N+L	Negative	Positive
42	235197	32	M	Severe AS, severe AR, moderate MS, moderate MR, moderate TR, PH	Negative	Negative	11,000	10	50	√ L+N	Negative	Positive
43	234977	16	M	Severe MS, severe MR, moderate TR	Negative	Positive	14,900	10	50	Absent	Positive	Positive
44	236358	50	M	Severe MS ,mild MR, mild AR, severe AR	Negative	Positive	8,600	08	50	√ N+L	Positive	Negative
45	237021	55	M	RHD, severe MS, mild MR,	Negative	Negative	5,600	48	30	√ L	Positive	Negative
46	251807	38	F	Severe MR	Negative	Negative	9,400	4	20	√ L	Negative	Negative
47	255713	23	M	RHD, severe MR/TR/AR	Negative	Negative	10,600	06	40	√ L	Positive	Negative
48	257336	44	F	Severe MR, severe MS, PH, TR/AF	Negative	Negative	7,800	08	50	Absent	Positive	Negative
49	259139	30	M	Severe MS/MR/AS/AR	Negative	Negative	8,900	12	40	Absent	Positive	Positive
50	258021	56	M	Severe MS, severe AS	Negative	Negative	4,700	28	40	√ L	Negative	Negative
51	258016	50	F	Severe MR, severe MS	Negative	Negative	7,800	5	60	Absent	Negative	Negative
52	232571	40	M	Severe MS, moderate MR, moderate TR,AR	Negative	Negative	8,700	2	50	Absent	Negative	Positive
53	91744	54	M	Severe MR, moderate MS, severe AR, mild TR, PH	Negative	Positive	8,100	8	40	Absent	Negative	Negative
54	236549	42	F	Moderate – MS MR	Negative	Negative	11,000	10	30	√ N+L	Negative	Negative
55	236936	24	F	Moderate - MS MR	Negative	Negative	7,200	6	30	√ L+N	Positive	Positive
56	237021	55	M	Severe MS	Negative	Negative	5,600	48	20	Absent	Negative	Negative
57	238881	38	F	Severe MR, moderate MS	Positive	Negative	7,000	25	20	Absent	Negative	Negative
58	239292	47	F	MS,AR	Negative	Positive	6,900	16	20	Absent	Negative	Negative
59	240784	50	M	Severe MS, severe PH, mild TR	Negative	Positive	5,300	12	40	Absent	Negative	Negative
60	239308	37	F	Severe MS, severe TR/PH, ASD, AF	Negative	Negative	9,600	10	65	Absent	Negative	Negative
61	244572	28	F	Severe MS, PH, TR	Negative	Negative	7,800	8	40	Absent	Negative	Not done
62	248954	35	F	Severe MS, moderate MR, mild TR, PH	Negative	Negative	15,800	15	40	Absent	Negative	Not done
63	251093	41	F	Severe MR, severe AR, trivial TR	Negative	Negative	7,900	18	30	Absent	Positive	Positive
64	291956	40	M	RHD, severe MR,AF	Negative	Negative	9,200	4	60	Absent	Negative	Negative
65	296775	50	F	Severe MR, PH	Negative	Negative	10,200	4	20	Absent	Positive	Positive
66	303143	22	M	RHD, severe MR, moderate MS, severe TR	Negative	Positive	7,400	4	50	Absent	Negative	Not done
67	302845	22	M	RHD, severe MR, moderate MS	Negative	Negative	6,800	6	60	√ L+N	Positive	Positive
68	258963	25	M	Severe MS, severe MR	Negative	Negative	6,200	8	50	√ L+N+E severe	Positive	Positive
69	260814	20	F	RHD, severe MR, mild AR	Negative	Negative	7,800	10	20	Absent	Positive	Negative
70	262165	45	F	Severe MR, mild AR, moderate TR, PH	Negative	Negative	10,200	20	30	√ L+N	Negative	Negative

Slide No.	Hosp. No.	Age	Sex	Diagnosis	ASO	CRP	TC	ESR mm/hr	Cellularity (%)	Inflammation	EV RNA NISH	IHC
71	277912	42	M	Severe AS, moderate AR, moderate MS, mild MR	Negative	Negative	12,200	6	20	√L	Positive	Negative
72	282000	48	M	Severe AS, mild AR, moderate MR, mild MS	Negative	Negative	8,500	5	10	Absent	Positive	Positive
73	281656	40	F	Severe MR, mild AR, moderate TR	Negative	Positive	10,900	18	5	Absent	Positive	Positive
74	287282	40	F	Mild MS, mild AS, severe TR	Negative	Positive	7,100	5	5	Absent	Positive	Positive
75	289260	28	M	RHD, severe MS, mild MR, moderate AR, severe TR, PH	Negative	Positive	7,100	20	70	√ L	Negative	Positive
Mean		31.5					17,900	15	40			
SD		11.32					1,665.99	6.49	18.02			

L: Lymphocytes; P: Plasma cells; N: Neutrophils; E: Eosinophils; RHD: Rheumatic Heart Disease; MS: Mitral Stenosis; MR: Mitral Regurgitation; TR: Tricuspid Regurgitation; AR: Aortic Regurgitation; AS: Aortic Stenosis; AF: Atrial Fibrillation; PH: Pulmonary Hypertension; ASD: Atrial Septal Defect.