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Improved diagnosis of cat-scratch disease with an IgM enzyme-linked immunosorbent assay for *Bartonella henselae* using *N*-lauroyl-sarcosine-insoluble protein antigen

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To the Editor,

Cat-scratch disease (CSD) is a frequent worldwide zoonosis caused by *Bartonella henselae* and characterized by regional lymphadenopathy and often fever and malaise. The detection of *B. henselae* DNA by polymerase chain reaction (PCR) is considered the "gold standard" for diagnosis, but requires invasive procedures to obtain lymph node or pus specimens [1]. Thus, diagnosis relies on indirect serologic testing with immunofluorescence assay (IFA), which is limited by subjective interpretation, elaborate test procedures with a low throughput, and relatively low sensitivity (IgM-IFA: 54%-72% [2, 3]; IgG-IFA: 62%-85% [2]). As an alternative to IFA, several enzyme-linked immunosorbent assays (ELISAs) have been developed with various *B. henselae*-specific antigens: whole-cell proteins, water-insoluble protein, recombinant 17-kDa protein, GroEL, outer membrane protein, and *N*-lauroyl-sarcosine-soluble protein (reviewed in **Supplementary Material, Table S1**). However, the sensitivity of most of these ELISAs was limited in PCR-positive CSD patients **(Supplementary Material, Table S1)**. Interestingly, an IgM-ELISA using refined *N*-lauroyl-sarcosine-insoluble protein (sarcosine-insoluble protein) showed improved sensitivity (83%) in CSD patients diagnosed by IgM-IFA [3].

Here, we compared diagnostic performance of new in-house assays using sarcosine-insoluble *B. henselae* antigens, such as IgM-ELISA, IgG-ELISA, and IgM/IgG/IgA-ELISA ("screening test"), to conventional IgM-IFA and IgG-IFA in a unique cohort of patients with CSD confirmed by PCR in lymph node or pus specimens, and in a clinically relevant control group.

Twenty-five patients of a previous cohort [4] with a positive *B. henselae*-specific PCR result from lymph node or pus specimens and additional sera stored at −20°C for further testing were included. Control samples derived from a control cohort of a previous study [1]

consisting of 23 blood donors from Zurich. The local ethics committee approved the protocol for this study (2017-01421).

For the ELISA, the sarcosine-insoluble protein was prepared from *B. henselae* reference strain (ATCC 49882) cultures on chocolate agar plates as described previously by Otsuyama et al. (antigen IV) [3]. Western blot analysis using IgM-IFA- and IgG-IFA-positive sera confirmed a band in the region of 8-10 kDa for sarcosine-insoluble proteins of *B. henselae*, and a complete lack of nonspecific reactions with IFA-negative sera **(Supplementary material, Figure S1)**. The ELISAs were performed as described previously [3], with some modifications. In brief, 120 µl of antigen diluted in 50 mM carbonate-bicarbonate buffer (pH 9.2) (IgM-ELISA and IgG-ELISA, 1:80; IgM/IgG/IgA-ELISA, 1:100) were coated on 96-well half-area polystyrene plates (Sigma, St. Louis, MO, USA) o/n at 4°C. After blocking with PBS/5% skim milk/0.1% Tween 20 for 4 h at RT, 110 µl of sample diluted in PBS/0.1% Tween 20 (PBS-T) (test sera, 1:100; control sera, 1:100, 1:200, 1:400, and 1:800) were incubated in duplicate for 1 hour at 37°C. For IgM-ELISA, samples were diluted in anti-human IgG solution (1:10; Virion\Serion, Würzburg, Germany) followed by PBS-T (test sera, 1:10; control sera, 1:2, 1:4, 1:8, 1:16; final dilution: test sera, 1:100; control sera, 1:20, 1:40, 1:80 and 1:160). Goat anti-human peroxidase detection antibodies (IgM, IgM-ELISA; IgG, IgG-ELISA; polyvalent Igs [G, A, M], IgM/IgG/IgA-ELISA; all Sigma) were diluted in PBS-T (IgM-ELISA, 1:500; IgG-ELISA and IgM/IgG/IgA-ELISA, 1:1,000), added, and binding measured using o-phenylendiamine (Sigma) as optical density (OD) at 490 nm. IgM-IFA- and IgG-IFA-positive sera were used to determine the arbitrary units (AU) of anti-*B. henselae* antibodies **(Supplementary material, Figure S2)**.

Detection of anti-*B. henselae* antibodies by IFA was performed as described previously [2] with commercial slides with host cell-free agar-derived *B. henselae* for IgM (MRL Diagnostics, Cypress, CA, USA) using a cutoff of 1:20, and with commercial slides with Vero cell-associated *B. henselae* for IgG (MRL Diagnostics) using a cutoff of 1:256.

The best cutoff of the ELISA in differentiating between PCR-positive CSD patients and control subjects was defined as the optimal threshold that maximized the distance to the identity (diagonal) line in the receiver operating characteristic (ROC) curve according to Youden J statistic. Area under the ROC curve (AUC) differences were calculated using the DeLong test. All reported *p*-values are 2-tailed with statistical significance defined as *p*-value <0.05. Data were analyzed using the R software environment (version 3.6.0).

The IgM-ELISA, IgG-ELISA, and IgM/IgG/IgA-ELISA yielded positive results in 24 (96%), 18 (72%), and 19 (76%) of PCR-positive CSD patients, using the defined optimal cutoffs of 0.10 AU, 1.00 AU and 0.57 AU, respectively **(Figure 1A-C)**. Control samples with positive results were found by IgM-ELISA in 2 (9%), IgG-ELISA in 8 (35%), and IgM/IgG/IgA-ELISA in 5 (22%) cases. Thus, the diagnostic odds ratio was significantly higher for IgM-ELISA than for IgG-ELISA ($p<0.01$) and IgM/IgG/IgA-ELISA ($p<0.01$) **(Supplementary material, Table S2)**. Compared to IFA, the IgM-ELISA showed a higher sensitivity with 0.96 (95% CI, 0.80-0.99) than IgM-IFA (0.88, 95% CI, 0.70-0.96; $p=0.61$) and IgG-IFA (0.84, 95% CI, 0.65-0.94; $p=0.35$). In contrast to IgM-ELISA with 2 (9%) false-positive results, the specificities of IFAs were 100% **(Figure 1D-E)**. Three (12%) CSD patients were IgM-positive but IgG-negative (ELISA and IFA), whereas only 1 (4%) patient was IgG-positive but IgM-negative (ELISA and IFA). Overall, the AUC was better for IgM-ELISA (0.96, 95% CI, 0.91-1.00) than IgM-IFA (0.94, 95% CI, 0.88-1.00; $p=0.41$) and IgG-IFA (0.92, 95% CI 0.84-1.00; $p=0.44$) **(Figure 1F)**.

In conclusion, we corroborate previous findings about the IgM-ELISA using sarcosine-insoluble *B. henselae* antigen [3] in a more relevant cohort of PCR-confirmed CSD patients. This IgM-ELISA is indeed a sensitive (96%) and specific (91%) test for the serodiagnosis of *B. henselae* infection. IgM can be detected up to 3 months following *B. henselae* infection [2]

and enables diagnosis prior to a seroconversion of IgG (12%). A limitation of the study is the small control group and that we did not test for cross-reactivity of IgM antibodies, as it has previously been described [1, 5]. The IgM-ELISA may overcome IFA in the routine diagnosis of CSD due to its objective, automated, and high-throughput analyses in addition to the excellent diagnostic performance.

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Author contributions

J.W. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: all authors;

Acquisition of data: J.W., P.M.M.S., R.Z.;

Analysis and interpretation of data: all authors;

Drafting of the manuscript: P.M.M.S.;

Critical revision of the manuscript for important intellectual content: all authors;

Study supervision: C.B.

Conflict of interest

None.

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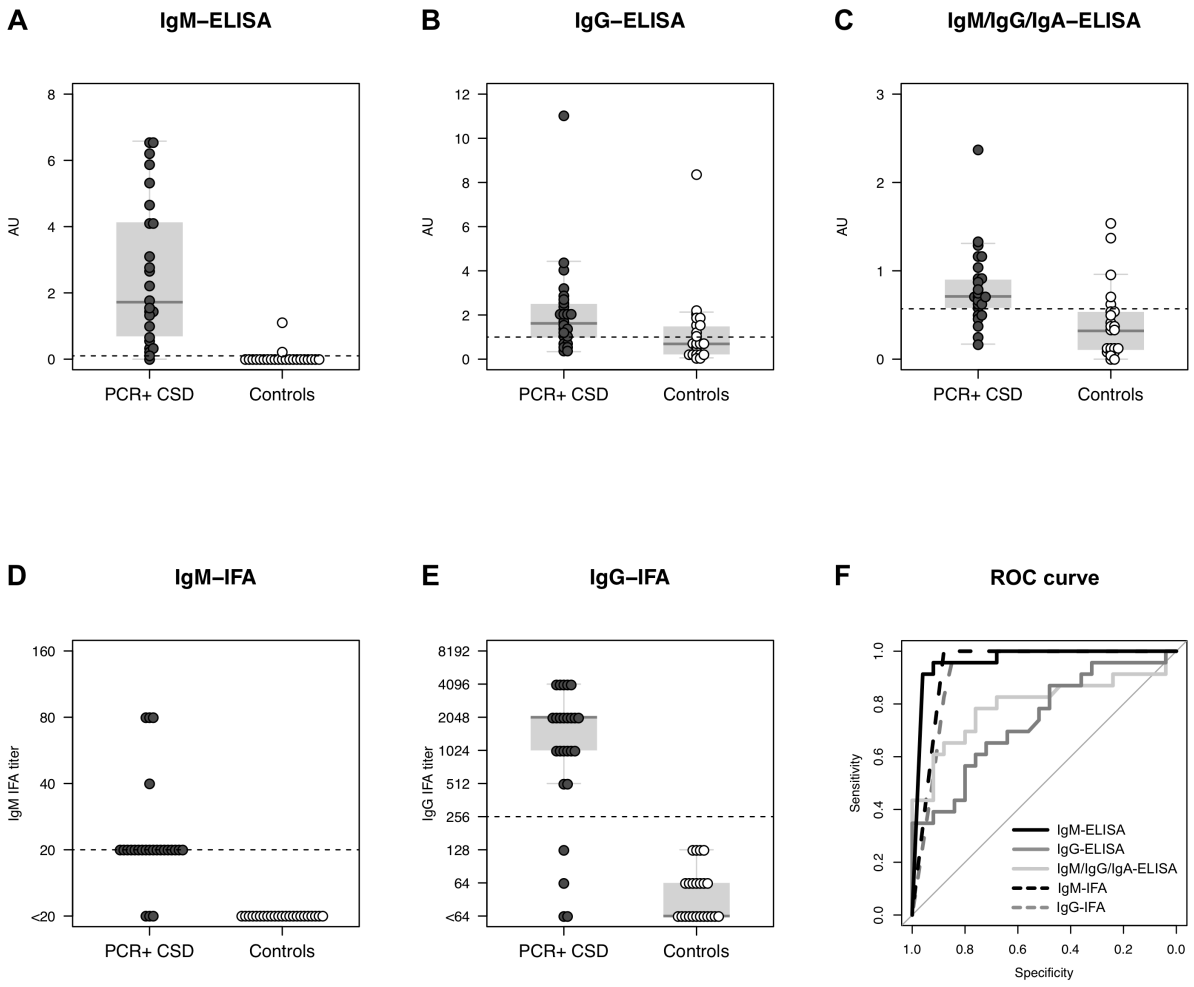
Figures

Figure 1. (A-E) Comparison of *Bartonella henselae*-specific ELISA **(A-C)** and IFA **(D-E)** test results between PCR-positive CSD patients (*n*=25) and control subjects (*n*=23). The median is shown as a dark gray line across the gray box that represents the lower and upper quartiles. Whiskers extend to the maximum or minimum values within 1.5 times the interquartile range above and below the 3rd and 1st quartile, respectively. The dashed line represents the optimal cut-off of the test that maximizes the distance to the identity (diagonal) line in the ROC curve (Figure 1F) according to Youden J statistic using the "coords" function in R software environment (version 3.6.0). Differences in proportions (Fisher exact test) and medians (Mann-Whitney *U* test) were statistically significant for all tests between groups ($p<0.01$). **(F)** Receiver operating characteristics (ROC) curve of sensitivity vs. specificity for *B. henselae*-specific ELISA and IFA. Statistically significant area under the ROC curve (AUC) differences (DeLong's test): IgM-ELISA vs. IgG-ELISA, $p<0.01$; IgM-ELISA vs. IgM/IgG/IgA-ELISA, $p=0.02$; IgG-ELISA vs. IgM-IFA, $p=0.02$; IgG-ELISA vs. IgG-IFA, $p=0.01$. Abbreviations: AU, arbitrary units; AUC, area under the receiver operating characteristic curve; CSD, cat-scratch disease; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; Ig, immunoglobulin; PCR, polymerase chain reaction; ROC, receiver operating characteristics.


A
IgM−ELISA
AU
PCR+ CSD
Controls
B
IgG−ELISA
AU
PCR+ CSD
Controls
C
IgM/IgG/IgA−ELISA
AU
PCR+ CSD
Controls
D
IgM−IFA
IgM IFA titer
<20
PCR+ CSD
Controls
E
IgG−IFA
IgG IFA titer
<64
PCR+ CSD
Controls
F
ROC curve
Sensitivity
Specificity
IgM-ELISA
IgG-ELISA
IgM/IgG/IgA-ELISA
IgM-IFA
IgG-IFA

165

Supplementary Material

Figures

Figure S1. Western blot analysis of the prepared sarcosine-insoluble *Bartonella henselae* protein antigen. Lane a, marker; lane b, IgM-IFA- and IgG-IFA-positive sera derived from PCR-positive CSD patients; lane c, IFA-negative control sera. Molecular size markers (kDa) are indicated on the left. Serum samples were tested as previously described [3], with some modifications. Briefly, the antigen was diluted in the loading buffer Roti®-Load (Carl Roth, Karlsruhe, Germany). For separation we used a 4-12% Bis-Tris gel (Expedeon, San Diego, CA, USA) for SDS-PAGE with 2-MES (pH 7.3) (Sigma, Missouri, USA) as running buffer. The membranes were stained with Ponceau S (Sigma, Missouri, USA), while polyclonal goat anti-human polyvalent Igs (IgM, IgG, IgA)-peroxidase antibody (Sigma, Missouri, USA) was used as the antibody and Super Signal West Dura substrate (Thermo Fisher Scientific, Massachusetts, USA) was used as the chemiluminescent substrate for the Western blotting.

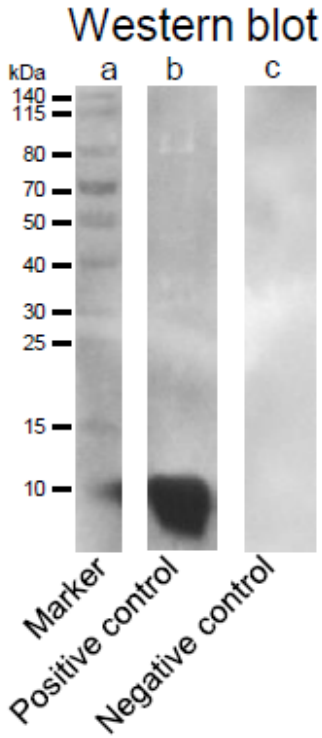
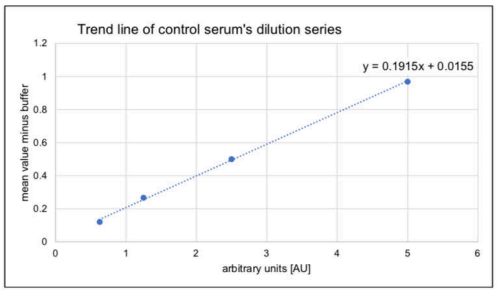


Figure S2. Transformation from optical density (OD) to arbitrary unit (AU) separately for each ELISA. OD results of a dilution series of IgM-IFA- and IgG-IFA-positive sera were assigned to AU.

IgM-ELISA:

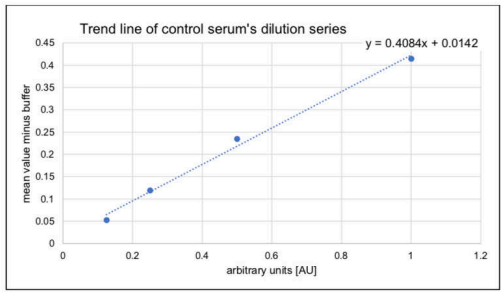
Dilution series of control serum	OD value 1	OD value 2	mean value	mean value minus buffer	arbitrary units
1:20	0.91	1.089	0.9995	0.96835	5
1:40	0.497	0.567	0.532	0.50085	2.5
1:80	0.275	0.323	0.299	0.26785	1.25
1:160	0.138	0.165	0.1515	0.12035	0.625



$$x[AU] = \frac{y - 0.0155}{0.1915} = \frac{mean\ value\ of\ tested\ serum - buffer - 0.0155}{0.1915}$$

IgG-ELISA:

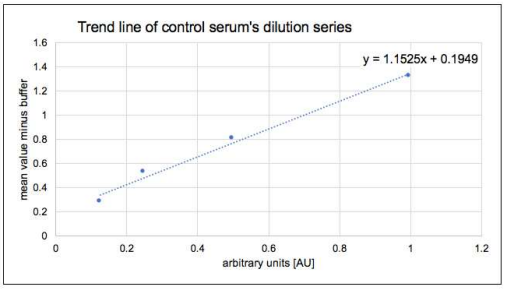
Dilution series of control serum	OD value 1	OD value 2	mean value	mean value minus buffer	arbitrary units
1:100	0.413	0.436	0.4245	0.415	1
1:200	0.23	0.259	0.2445	0.235	0.5
1:400	0.127	0.131	0.129	0.1195	0.25
1:800	0.053	0.072	0.0625	0.053	0.125



$$x[AU] = \frac{y - 0.0142}{0.4084} = \frac{mean\ value\ of\ tested\ serum - buffer - 0.0142}{0.4084}$$

IgM/IgG/IgA-ELISA:

Dilution series of control serum	OD value 1	OD value 2	mean value	mean value minus buffer	arbitrary units
1:100	1.384	1.317	1.3505	1.3235	1
1:200	0.861	0.817	0.839	0.812	0.5
1:400	0.575	0.534	0.5545	0.5275	0.25
1:800	0.338	0.271	0.3045	0.2775	0.125



$$x[AU] = \frac{y - 0.1949}{1.1525} = \frac{mean\ value\ of\ tested\ serum - buffer - 0.1949}{1.1525}$$

Tables

Table S1. Overview of diagnostic performances of *Bartonella henselae*-specific ELISAs in the literature.

Reference	Otsuyama et al. J Clin Microbiol 2016;54:1058-64	Jost et al. J Clin Microbiol 2018;56:e01329-18	Ferrara et al. Lett Appl Microbiol 2014;59:253-62	Tsuruoka et al. Diagn Microbiol Infect Dis 2012;74:230-5	Hoey et al. Clin Vaccine Immunol. 2009;16:282-4	Herremans et al. Eur J Clin Microbiol Infect Dis 2009;28: 147-52	Herremans et al. J Microbiol Methods 2007;71:107-13	Vermeulen et al. Clin Microbiol Infect 2007;13:627-34	Loa et al. Diagn Microbiol Infect Dis 2006;55:1-7	Litwin et al. Am J Clin Pathol 1997;108:202-9	Szelc-Kelly et al. Pediatrics 1995;96:1137-42
Antigen:											
- Whole-cell antigen						+	+	+			+
- N-lauroyl-sarcosine-insoluble protein	+ ("antigen IV")										
- N-lauroyl-sarcosine-soluble protein				+							
- Water-insoluble protein		+ ("fraction 24")									
- Recombinant 17-kDa protein			+		+				+		
- GroEL			+								
- Outer membrane protein										+	
Samples with test criteria:											
- *CSD-positive, n:*	24	43	64	118	13	126	155	51	45	131	56
- PCR-positive, *n*	**8***	**10****	–	–	–	**126**	**155**	**51**	–	–	–
- IFA-positive (IgG and/or IgM), *n*	24	33	64	118	13	–	–	–	25	131	–
- Skin test-positive, *n*	–	–	–	–	–	–	–	–	–	–	56
- *Controls, n:*	85	16	87	88	34	126	244	56	86	10	57
- PCR-negative, *n*	–	–	–	–	–	–	–	56	–	–	–
- IFA-negative, *n*	85	16	87	88	34	–	–	–	86	10	–
ELISA performance:											
- IgM:											
- Sensitivity (%)	83	–	86 (17-kDa) 98 (GroEL)	–	100	56	32	65	–	94	73
- Specificity (%)	100	–	75 (17-kDa) 45 (GroEL)	–	97	98	98	91	–	99	95
- IgG:											
- Sensitivity (%)	–	100 (PCR+) 76 (IFA+)	76 (17-kDa) 82 (GroEL)	96	–	36	45	–	71	86	18
- Specificity (%)	–	93 (PCR+) 93 (IFA+)	66 (17-kDa) 43 (GroEL)	98	–	98	98	–	93	96	95
IFA performance:											
- IgM:											
- Sensitivity (%)	54	–	–	–	–	–	–	53	–	–	–
- Specificity (%)	–	–	–	–	–	–	–	93	–	–	–
- IgG:											
- Sensitivity (%)	–	–	–	–	–	–	–	–	–	–	93
- Specificity (%)	–	–	–	–	–	–	–	–	–	–	98

*8 out of 24 IFA-positive CSD patients were also tested by PCR (lymph node, *n*=4; whole blood, *n*=4); **10 out of 43 CSD patients were tested by PCR (lymph node, *n*=10). Test characteristics and criteria for samples as in the current report are indicated in dark gray.

Table S2. Diagnostic accuracy for *Bartonella henselae*-specific ELISA and IFA.

Test	Sensitivity (95% CI)	Specificity (95% CI)	LR– (95% CI)	LR+ (95% CI)	DOR (95% CI)
IgM-ELISA	0.96 (0.80–0.99)	0.91 (0.73–0.98)	0.04 (0.01–0.30)	11.04 (2.93–41.60)	252.00 (21.30–2981.84)
IgG-ELISA	0.72 (0.52–0.86)	0.65 (0.45–0.81)	0.43 (0.21–0.86)	2.07 (1.12–3.81)	4.82 (1.42–16.40)
IgM/IgG/IgA-ELISA	0.76 (0.57–0.89)	0.78 (0.58–0.90)	0.31 (0.15–0.64)	3.50 (1.56–7.83)	11.40 (2.95–44.00)
IgM-IFA	0.88 (0.70–0.96)	1.00 (0.86–1.00)	0.12 (0.04–0.35)	NA	NA
IgG-IFA	0.84 (0.65–0.94)	1.00 (0.86–1.00)	0.16 (0.07–0.39)	NA	NA

Abbreviations: CI, confidence interval; DOR, diagnostic odds ratio; LR-, negative likelihood ratio; LR+, positive likelihood ratio; NA, not available.