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Manuscript Number: EJCMIID-D-10-00080R1

Title: Use of recombinant chimeric antigens for serodiagnosis of Mycoplasma pneumoniae infection

Article Type: Article

Keywords: Mycoplasma pneumoniae; diagnosis; recombinant antigens; enzyme-linked immunosorbent assay; ELISA

Corresponding Author: Dr. Francesca Montagnani, M.D, Ph.D.

Corresponding Author's Institution: Clinica di Malattie Infettive, Università di Siena

First Author: Francesca Montagnani, M.D, Ph.D.

Order of Authors: Francesca Montagnani, M.D, Ph.D.; Francesca De Paolis; Elisa Beghetto; Nicola Gargano

Abstract: Purpose. We have evaluated the diagnostic utility of three antigenic regions of the Mycoplasma pneumoniae P1, P30, and MNP456 gene products in order to replace the soluble, whole-cell bacterial extract in serological assays. Antigenic regions, being previously identified B cell epitopes, were used individually or assembled in a recombinant chimeric antigen by genetic engineering. Methods. Paired serum samples from 47 patients with M. pneumoniae infection and from 39 subjects with a clinical picture of atypical pneumonia but without a defined diagnosis of M. pneumoniae infection, were included. Immunoglobulin G (IgG) antibodies against epitopes carried by recombinant antigens were measured by performing recombinant enzyme-linked immunosorbent assays (Rec-ELISA's). Rec-ELISA's results were compared to those obtained by a commercial assay using the whole-cell Mycoplasma antigen. Results. Our study demonstrates that all IgG Rec-ELISA's using recombinant antigens have better sensitivity with respect to the commercial assay. Furthermore, we show that the use of chimeric antigens improve the performance of the assays. Conclusions. The use of recombinant antigens is effective in distinguishing M. pneumoniae-infected from uninfected individuals and shows that immunoassays based on recombinant antigens could provide the basis for standardized commercial tests for the serodiagnosis of M. pneumoniae diseases.

Response to Reviewers: RE: Ms. No. EJCMIID-D-10-00080

"Use of recombinant chimeric antigens for serodiagnosis of Mycoplasma pneumoniae infection"

Dear Prof. Van Belkum,

Thank you very much for the comments to our paper. The manuscript has now been revised in accordance with the suggestions of the reviewers. We complied with all points raised and we believe that our manuscript is now more clear and complete.

Best regards,
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1) Abstract lines 5-12. Sentence should be: We have evaluated,.....gene products in order to replace the soluble, whole -cell bacterial extract in serological assays. Antigenic regions, being previously identified B cell epitopes, were used individually or assembled in a recombinant chimeric antigen by genetic engineering.

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We agree with the reviewer observation. For this reason serum samples from patients infected with *S. pneumoniae* have been used as negative controls and none of them did react with the recombinant antigens. Unfortunately, blood samples from patients with other non-respiratory mycoplasma species were not available at the time of the study.

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Materials and Methods: Line 5 -37, cloning of the chimeric antigen EC12. Location of the antigenic regions used: please refer to figure 1. PCR programs used? DNA extraction method? PCR reaction mixture? Please provide more details on pGEX-P30. Where does it come from? Cloning method? Briefly.

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ORIGINAL ARTICLE

TITLE: Use of recombinant chimeric antigens for serodiagnosis of *Mycoplasma pneumoniae* infection

Francesca Montagnani, Francesca De Paolis, Elisa Beghetto, Nicola Gargano

F. Montagnani (**Corresponding author**)

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F. De Paolis, E. Beghetto, N. Gargano

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Present affiliation: Sigma-tau Industrie Farmaceutiche Riunite s.p.a.,

Via Pontina Km 30.400,

00040 - Rome, Italy

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4 **ABSTRACT**
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8 **Purpose.** We have evaluated the diagnostic utility of three antigenic regions of the
9 *Mycoplasma pneumoniae* P1, P30, and MNP456 gene products; ~~used individually or further~~
10 ~~assembled in a recombinant chimeric antigen by genetic engineering~~, in order to replace the
11 soluble, whole-cell bacterial extract in serological assays. Antigenic regions, being previously
12 identified B cell epitopes, were used individually or assembled in a recombinant chimeric
13 antigen by genetic engineering.
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19 **Methods.** Paired serum samples from 47 patients with *M. pneumoniae* infection and from 39
20 subjects with a clinical picture of atypical pneumonia but without a defined diagnosis of *M.*
21 *pneumoniae* infection, were included. Immunoglobulin G (IgG) antibodies against epitopes
22 carried by recombinant antigens were measured by performing recombinant enzyme-linked
23 immunosorbent assays (~~Rec-ELISAs~~Rec-ELISA's). ~~Rec-ELISAs~~Rec-ELISA's results were,
24 ~~and~~ compared to ~~results those~~ obtained by a commercial assay ~~with using~~ the whole-cell
25 *Mycoplasma* antigen.
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34 ~~Rec-ELISAs using recombinant antigens have performance characteristics~~sensitivity
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36 that the use of chimeric antigens improve the performance of the assays.
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43 infected from uninfected individuals and shows that immunoassays based on recombinant
44 ~~products~~antigens could provide the basis for standardized commercial tests for the
45 serodiagnosis of *M. pneumoniae* diseases.
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Keywords: *Mycoplasma pneumoniae*, diagnosis, recombinant antigens, enzyme-linked immunosorbent assay, ELISA

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4 **INTRODUCTION**
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8 *M. pneumoniae* is the leading cause of atypical pneumonia in children and young
9 adults; it is responsible for 10-20% of cases of community acquired pneumonia, giving rise to
10 extrapulmonary complications in 25% of infected patients[1-3].
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13 Infected individuals show flu-like symptoms, but characteristically there is an
14 insidious onset with chronic recovery [4,2]. Misdiagnosis and subsequent inappropriate
15 antibiotic treatment pose a serious threat to infected individuals [5,6]. Although clinicians
16 may suspect infections during epidemics, an aetiological diagnosis cannot be formulated on
17 the basis of clinical appearance, since other bacterial and viral infections often share
18 symptoms and clinical features [7,2].
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24 Diagnosis of *M. pneumoniae* infection can be established by isolating bacteria or
25 detecting *mycoplasma*-specific DNA sequences and immunoglobulins in body fluids and sera
26 from infected individuals, respectively. Serological methods play a predominant role in routine
27 clinical practice, mainly due to the ease of specimen collection and the widespread availability
28 of serologic tests [8].
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34 Host antibody response to *M. pneumoniae* infection can be detected after 1 week of
35 illness, but it generally peaks three to six weeks after acute infection, followed by a gradual
36 decay of antibody production over months to years [1,9]. As a result of the long incubation
37 period, the antibody response is often evident by the time symptoms appear. A simultaneous
38 testing for both IgG and IgM in paired serum sample collected 2 to 3 weeks apart is usually
39 required to achieve a proper diagnosis.
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46 Serodiagnosis of *M. pneumoniae* infection can be performed by use of complement
47 fixation tests (CF), indirect immunofluorescent assays (IFA) and enzyme-linked
48 immunoassays (EIA) to detect the presence of the various classes of anti-*Mycoplasma*
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4 immunoglobulins (IgG, IgM) [9]. All of the commercially available assays use the whole *M.*
5 *pneumoniae* soluble extract or sub-cellular fractions as the antigen. However, these assays
6
7 have limited sensitivity and specificity since cross-reactions of the antigen with antibodies to
8
9 other *Mycoplasma* species and other bacterial pathogens may occur [1]. Also, the available
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11 assays may vary in their ability to detect serum immunoglobulins [10,11], due to the lack of a
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13 purified standardized antigen or standard methods for preparing the antigen.
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16 The aim of this study was to improve the performance of enzyme-linked
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18 immunoassays for the serodiagnosis of *M. pneumoniae* infection using recombinant
19 ~~productsantigens~~, which were ~~challengedchallengetestested~~ with sera from a cohort of patients
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21 with respiratory tract diseases clinically compatible with “atypical” bacterial aetiology. [We](#)
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23 [have previously shown the identification and characterization of immunodominant B-cell](#)
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25 [epitopes of *M. pneumoniae* antigens using the phage-display technology \[12\]. We show here](#)
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27 [the usefulness of recombinant proteins containing epitopes of the P1, P30 and MPN456](#)
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29 [antigens in *M. pneumoniae* serodiagnosis.](#)
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MATERIALS AND METHODS

Cloning the chimeric antigen EC12

The DNA coding for the antigenic regions of *M. pneumoniae* P1 (residues 1307-1340), P30 (residues 194-241) and MPN456 (residues 765-846) open reading frames [12] were used for the construction of the chimeric antigen EC12. DNA fragments of P1 and MPN456 were amplified by PCR from *M. pneumoniae* DNA, FH strain [\(DNA commercially available and purchased from Abionline, USA\)](#), using specific oligonucleotides as follows:

P1-for, 5'-GGGGATCCTTCACATTGGCAGTGTGCTTAG-3';

P1-rev, 5'-AGGCTACCGCCACCAGAACTATCCCACCACCTCCA-3';

MPN456-for 5'-GTTCTGGTGGCGGTAGCCTGTTAGCTGCGAGTCAAAA-3';

MPN456-rev 5'-ACTAGTGCTACCGCCACCAGATGCTTTAGCATCACCAAAGTTC-3'.

[PCR conditions were 30 sec at 94°C, 30 sec at 55°C and 60 sec at 72°C for 30 cycles.](#) PCR products of P1 and MPN456 fragments were purified, mixed and then used as templates for DNA amplification using oligonucleotides P1-for and MPN456-rev. [The PCR protocol was 30 sec at 94°C, 30 sec at 55°C and 60 sec at 72°C for 25 cycles.](#) The resulting DNA was purified [by standard methods](#) and cloned into the plasmid pGEX-P30 [12], [which contained](#) the antigenic region of the P30 [protein-antigen \(amino acids 194-241\) fused at the N-terminus of the GST protein\[42\]](#), to generate the plasmid pGEX-EC12 [\(schematically shown in Fig. 1\)](#).

Purification of recombinant antigens

Four *M. pneumoniae* antigens were used: P1, P30, MPN456 [13] and EC12. Recombinant antigens were expressed in bacterial cells as fusion proteins with glutathione S-transferase (GST) and purified by affinity chromatography as described previously [13].

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4 Briefly, recombinant *Escherichia coli* strain AD202 were grown in Luria Bertani (LB)
5 medium and gene expression was induced with using isopropyl-β-D-thiogalactopyranoside
6 (IPTG). Bacteria were centrifuged at 2000 x g for 30 min at 4°C and then suspended in STE
7 buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) containing 100 μg mL⁻¹ of lysozyme and
8 protease inhibitors (Boehringer, Germany). The mixture was sonicated, and Triton X-100 was
9 added to a final concentration of 1%. After centrifugation at 10,000 x g for 30 min at 4°C, the
10 supernatant was incubated with glutathione-Sepharose beads (BD Biosciences, USA), and the
11 GST-fusion proteins were eluted following the manufacturer's instructions and stored at -
12 20°C.
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21 The recombinant antigens were efficiently expressed and purified in large amounts
22 from the cytoplasm of bacterial cells, with a purity >90% and a yield of purified proteins of 5-
23 15 mg per liter of bacterial cultures.
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28 Serum samples

29 One hundred eighty-four serum samples collected from 98 patients hospitalized for
30 pulmonary diseases at the Clinic of Infectious Diseases of Siena University, Italy, during
31 years 1999 to 2005, were used. A clinical picture compatible with atypical pneumonia [2]
32 was defined by the presence of the following features: insidious onset, malaise, fever, cough
33 ≥ 1 week; signs of interstitial pneumonia ± wheezing; extra-pulmonary manifestations
34 compatible with *Mycoplasma* infection (e.g. arthralgia, skin eruption); radiological findings
35 suggestive of interstitial involvement ± minimal pleuritis; lack of responsiveness to previous
36 beta-lactams therapy.
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45 Accordingly to the results obtained by clinical and diagnostic examinations, serum
46 samples were divided into three groups as follows:
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- group A ($n=94$), was composed of samples from 42 paediatric patients (males, 52.4%; median age, 10 years; interquartile range [IQR], 8-12) and 5 adults (males, 20%; median age, 21 years; IQR, 20-28) who had a clinical picture compatible with atypical pneumonia and a defined diagnosis of *M. pneumoniae* infection by the CF test and/or PCR assays on pharyngeal swab or sputum;
 - group B ($n=78$), was composed of samples from 35 paediatric patients (males, 60%; median age 4 years, IQR 3-6) and 4 adults (males, 25%; median age, 24 years; IQR, 22-30), who had a clinical picture compatible with atypical pneumonia, but with: (i) a *M. pneumoniae* infection not previously defined (available methods with negative results or not performed); (ii) a direct or indirect diagnostic investigation for *Chlamydia pneumoniae* and respiratory viruses with negative results; (iii) no evidence of infection by other pulmonary bacterial pathogens (e.g. *Streptococcus pneumoniae*, *Haemophilus influenzae*);
 - group C ($n=12$), was composed of control sera from 12 paediatric patients affected by *Streptococcus pneumoniae* pneumonia and that resulted negative for *M. pneumoniae*-specific serology and PCR analysis.

35 Two sera were collected from each patient of groups (A) and (B); the first sample at the time
36 of recovery and the second after dismissing (usually 2-3 weeks after hospitalization).
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40 **Whole-cell *Mycoplasma* antigen immunoassays**

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42 Analysis of *Mycoplasma*-specific IgG antibodies were done by the whole-cell,
43 *Mycoplasma* antigen assay ELISA-IgG (IBL Hamburg, Germany), according to the
44 manufacturer's instructions ([e.g. sera diluted 1:100](#)). All serum samples were analyzed in a
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48 blinded fashion.
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4 **Recombinant protein enzyme immunoassays (Rec-ELISA)**
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6 Maxisorb plates (Nunc) were adsorbed with recombinant proteins at a concentration of
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8 10 µg mL⁻¹ in 50 mM NaHCO₃, pH 9.6. Each protein (GST-P1, GST-P30, GST-MPN456 and
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10 GST-EC12) was coated separately onto individual microtiter wells. After incubation
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12 overnight at 4°C, plates were blocked with 5% non-fat dry milk and 0.05% Tween 20 in PBS
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14 (blocking buffer) and subsequently incubated for 1 h at 37°C with serum samples diluted
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16 1:100 in blocking buffer. The plates were washed with 0.05% Tween-20 in PBS, and anti-
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18 human IgG alkaline phosphatase-conjugated antibodies (Sigma-Aldrich) were added to each
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20 well. Finally, incubating plates with the chromogenic substrate *p*-nitrophenylphosphate
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22 (pNPP; Sigma-Aldrich) revealed the enzymatic activity. The results were recorded as the
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24 difference between the absorbance (optical density, OD) at 405 nm and that at 620 nm, as
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26 detected by an automated ELISA reader (Labsystem Multiskan, Finland).

27 For each serum, the assay was done in duplicate and average values were calculated. For
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29 every GST-fusion product, the cut-off value was determined as the mean plus three times the
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31 standard deviation of the absorbance readings obtained from the *Mycoplasma*-negative sera
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33 (group C).
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36 **Statistical analysis**
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38 The sensitivity, specificity, positive and negative predictive values of the enzyme-
39
40 linked immunosorbent assays are given. Proportions were compared by the chi-square test or
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42 the Fisher exact test (two tailed), with a *p* value <0.05 considered statistically significant.
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4 **RESULTS**
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8 In a retrospective study, 184 sera from 98 patients hospitalized for pneumonia were
9 analyzed. Paired sera from 86 cases of atypical pneumonia were available and were further
10 divided into two groups, as follows: group (A), from 47 subjects with a defined diagnosis of
11 *M. pneumoniae* infection, group (B), from 39 subjects without a defined diagnosis of *M.*
12 *pneumoniae* infection but with a clinical picture compatible with atypical pneumonia. Twelve
13 sera from 12 patients affected by *Streptococcus pneumoniae* pneumonia with negative results
14 for *M. pneumoniae*-specific serology and PCR analysis were also assayed as negative controls
15 (group C).
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23 To set up an immunoassay based on recombinant ~~products~~antigens, three regions of
24 the *M. pneumoniae* P1, P30 and MPN456 gene products, which had been shown previously to
25 contain B-cell epitopes [12], were chosen for the investigation. The antigen fragments were
26 produced in *E. coli* as GST fusion products (namely GST-P1, GST-P30 and GST-MPN456).
27 Also, the antigenic regions of P1, P30 and MPN456 proteins were assembled to give the
28 chimeric antigen GST-EC12 (Figure 1).
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35 The immunoreactivity of GST-P1, GST-P30, GST-MPN456 and GST-EC12 with IgG
36 antibodies in sera from patients with a defined diagnosis of *M. pneumoniae* infection (group
37 A) was determined. Each of the antigens was coated separately in individual wells and tested
38 in the IgG ~~Rec-ELISAs~~Rec-ELISA's with paired sera from the 47 subjects of group A (Table
39 1). Overall, the proportion of IgG-reactive sera were 54%, 48%, 25% and 57% by use of
40 recombinant GST-P1, GST-P30, GST-MPN456 and GST-EC12 antigens, respectively. None
41 of the *Mycoplasma*-negative sera reacted with the GST fusion products. GST-MPN456, GST-
42 P1, GST-P30 and GST-EC12 specifically reacted with IgG antibodies present in at least one
43 of the paired sera from 40%, 87%, 81% and 94% of infected patients, respectively. Also, 40
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4 out of 47 patients (85%) developed specific IgG antibodies against at least two distinct *M.*
5 *pneumoniae* antigens along hospitalization.
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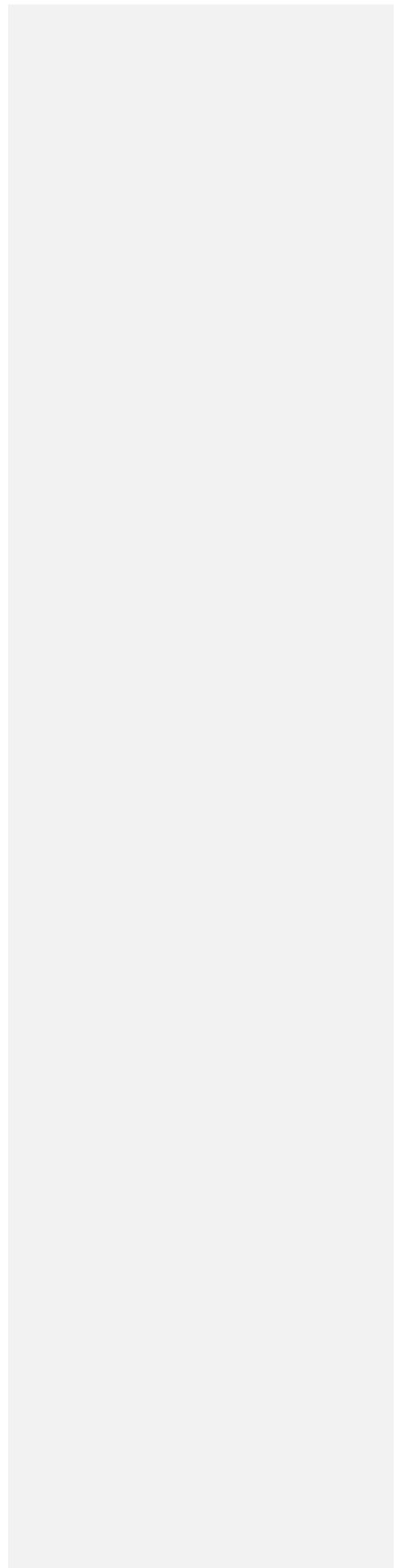
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8 Sera from group A patients were also analyzed with a commercial IgG ELISA assay,
9 which employs the whole cell extract of *M. pneumoniae* as the antigen (Table 1). A low
10 proportion of IgG-positive sera was observed (19%), allowing to obtain a correct diagnosis of
11 *M. pneumoniae* infection in only 17 out of 47 infected patients (36.2%). Also, only 12 out of
12 59 serum samples having specific anti-*Mycoplasma* antibodies, as assessed by the CF test,
13 were found to be positive with the commercial ELISA (patients A1-A39), allowing a positive
14 diagnosis in only 12 out of 39 subjects (31%).
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21 The performance of IgG assays employing the whole cell extract or recombinant
22 antigens for the serological diagnosis of *M. pneumoniae* infection is summarized in Table 2.
23 All of the assays based on recombinant antigens showed a significantly better performance
24 when compared to the commercial test. In particular, the assay based on the chimeric antigen
25 EC12 revealed a significantly higher sensitivity with respect to the commercial assay (93.6%
26 versus 36.2 %, respectively; $p < 0.001$).
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32 Next, the immunoreactivity of recombinant antigens was assessed with IgG antibodies
33 in sera from patient with a clinical picture of atypical pneumonia but without a defined
34 diagnosis of *M. pneumoniae* infection (group B), and the results of ~~Rec-ELISAs~~ Rec-ELISA's
35 were compared with those obtained using the commercial assay. Detailed results of all IgG
36 assays performed with paired sera collected from the 39 patients of group B are shown in
37 Table 3. Overall, 23 out of 78 sera specifically reacted with at least one recombinant antigen,
38 allowing serological diagnosis of *M. pneumoniae* infection in 13 patients (33%). In particular,
39 a constant IgG titre towards GST-EC12 and other recombinant antigens was revealed in 5
40 patients (B2, B28-B31), while an increase of IgG titre towards single antigens and/or EC12
41 was revealed in 3 patients (B32-B34) with a negative diagnosis of *M. pneumoniae* infection
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on the basis of the CF assay and in 5 patients (B35-B39) who were not checked for *M. pneumoniae* infection. By contrast, none of the sera displayed a positive result when the commercial assay was used.



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4 **DISCUSSION**
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8 *M. pneumoniae* infection occurs endemically and epidemically worldwide, hence it
9 should be considered in the differential diagnosis of upper and lower respiratory tract diseases
10 [1,2,9]. Moreover, *M. pneumoniae* colonization is believed to be strongly implicated in the
11 pathogenesis and exacerbation of asthma, and it seems of certain concern in other chronic
12 diseases [9, 15-17, 19-21]. The clinical management of this “atypical” pathogen requires
13 aimed therapy but a standardized and rapid diagnostic test is lacking, due to inherent
14 limitations of current microbiological, molecular and serological assays [9].
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21 The present study evaluated the diagnostic utility of serological assays employing
22 recombinant proteins, composed by distinct antigenic regions of *M. pneumoniae* gene
23 products. The main objective of our work was, therefore, the provision of “artificial antigens”
24 and their use for the development of selective diagnostic immunoassays. Three distinct
25 antigenic regions of *M. pneumoniae* proteins [12] were used individually (P1, P30 and
26 MNP456) or further assembled into a chimeric product (EC12). By using a standard bacterial
27 expression system, all recombinant antigens were purified under native condition in large
28 amounts and were found to have good solubility and elevated purification yield, which are
29 fundamental prerequisites for the commercial use of recombinant ~~products~~antigens.
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38 The immunoreactivity of EC12 as well as the single antigenic regions composing the
39 chimera was assessed with IgG antibodies present in sera from patient hospitalized for *M.*
40 *pneumoniae* infection. All IgG assays employing recombinant antigens showed excellent
41 sensitivity and specificity, with the EC12-based assay reaching the best sensitivity (93.6%),
42 based on the available sera used in this study. The results of the chimeric antigen
43 immunoassay were in agreement with those obtained using the CF assay since 92% of the
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4 patients who received a positive diagnosis by the CF analysis were found to be positive in the
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6 EC12 Rec-ELISA.

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8 ~~Because a “gold standard” for the serological diagnosis of *Mycoplasma* infection is~~
9 ~~currently unavailable, the~~The results of the CF test were also compared to those obtained with
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11 a commercial assay which utilizes the whole *M. pneumoniae* cell extract as the antigen.
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13 Surprisingly, only 31% of subjects having specific anti-*Mycoplasma* antibodies, as measured
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15 by the CF test, were found to be positive ~~with~~by the commercial ELISA.

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18 The immunoreactivity of recombinant antigens was then investigated with sera from
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20 patients without a defined etiological diagnosis of *M. pneumoniae* infection but with a clinical
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22 picture compatible with atypical pneumonia. Noteworthy, the presence of IgG towards EC12
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24 was revealed in serum samples from six patients who received a negative diagnosis of *M.*
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26 *pneumoniae* infection by the CF test. Moreover, IgG antibodies against the recombinant
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28 antigens were detected in sera from seven patients who were not checked for *M. pneumoniae*
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30 infection. By contrast, none of these sera positively reacted with the commercial ELISA, thus
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32 further highlighting the better sensitivity of assays based on recombinant antigens.

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34 Previous studies have demonstrated the clinical usefulness of recombinant antigens for the
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36 serological diagnosis of *M. pneumoniae* infection in humans [22-26]. Also, the use of
37
38 recombinant ~~products~~antigens has been evaluated for different types of infections, particularly
39
40 those regarding veterinary *Mycoplasma* species [27-29]. However, the composition of the
41
42 protein cocktail representative of the *M. pneumoniae* antigenic repertoire and usable for
43
44 antibody detection in humans remains an open question ~~requiring further investigations~~. The
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46 present study demonstrates that the use of recombinant antigens is effective in distinguishing
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48 *M. pneumoniae*-infected individuals from uninfected individuals and that assays with such
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50 antigens have improved performance characteristics ~~than compared to~~ those using the whole-

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cell bacterial antigen, thus providing the basis of standardized assays for the serodiagnosis of
M. pneumoniae diseases.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1

Immunoreactivity of recombinant antigens with IgG antibodies in sera from patients with *M. pneumoniae* infection (group A)

Patient	Age (years)	Serum sample	Days from first sample	PCR ^a	IgG CF ^b	IgG ELISA ^c	IgG Rec-ELISA ^d			
							GST-P1	GST-P30	GST-MP456	GST-EC12
A1	11	A1a	+ 12	n.d.	NEG	0.075	0.191	0.077	0.081	0.173
		A1b			1/128	0.222	2.942	0.972	2.469	2.774
A2	15	A2a	+ 21	n.d.	NEG	0.121	0.092	0.106	0.094	0.125
		A2b			1/128	0.538	0.760	2.990	0.092	2.809
A3	13	A3a	+ 17	n.d.	<1/512	0.175	1.308	0.579	0.122	0.815
		A3b			>1/102	0.202	2.354	1.174	0.567	1.713
A4	12	A4a	+ 15	n.d.	NEG	0.084	0.060	0.066	0.060	0.124
		A4b			>1/102	0.104	0.045	0.048	0.052	0.103
A5	12	A5a	+ 17	n.d.	<1/16	0.188	0.055	0.064	0.071	0.161
		A5b			1/512	0.468	2.257	0.398	2.923	2.942
A6	10	A6a	+ 36	n.d.	NEG	0.100	0.161	0.202	0.140	0.185
		A6b			1/64	0.564	1.949	2.958	0.088	2.459
A7	10	A7a	+ 16	n.d.	1/256	0.075	0.073	0.140	0.071	0.129
		A7b			1/1024	0.317	0.435	1.174	0.063	0.626
A8	12	A8a	+ 38	n.d.	NEG	0.073	0.073	0.075	0.069	0.120
		A8b			1/64	0.639	0.351	2.123	0.084	0.613
A9	9	A9a	+ 17	n.d.	NEG	0.105	0.064	0.066	0.069	0.128
		A9b			1/128	0.395	1.508	2.400	0.888	1.040
A10	11	A10a	+ 15	n.d.	1/512	0.301	0.477	0.140	0.076	0.294
		A10b			1/512	0.468	1.032	1.737	0.137	1.175
A11	7	A11a	+ 13	n.d.	NEG	0.562	0.112	0.124	0.093	0.157
		A11b			1/128	0.269	0.864	1.253	0.070	0.962
A12	5	A12a	+ 12	n.d.	>1/102	0.379	2.906	3.013	0.083	2.992
		A12b			>1/102	0.443	2.954	2.880	0.085	2.846
A13	10	A13a	+ 24	n.d.	NEG	0.077	0.056	0.055	0.059	0.110
		A13b			1/1024	0.234	2.527	0.115	0.990	1.832
A14	20	A14a	+ 16	n.d.	1/256	0.151	0.075	0.309	0.058	0.309
		A14b			>1/102	0.348	0.338	2.213	0.347	1.365
A15	2	A15a	+ 20	n.d.	NEG	0.179	0.093	0.076	0.069	0.136
		A15b			1/32	0.201	0.543	1.045	0.085	0.934
A16	1.3	A16a	+ 11	n.d.	1/512	0.086	0.054	0.052	0.053	0.118
		A16b			>1/102	0.126	0.519	0.683	0.061	1.181
A17	13	A17a	+ 14	n.d.	1/32	0.146	0.105	0.127	0.115	0.171
		A17b			>1/102	0.033	0.055	0.116	0.074	0.238
A18	10	A18a	+ 13	n.d.	>1/102	0.138	0.368	0.281	0.294	0.587
		A18b			>1/102	0.431	1.193	1.053	0.456	2.263
A19	12	A19a	+ 16	n.d.	1/64	0.240	0.077	0.240	0.062	0.209
		A19b			1/128	0.219	0.333	1.115	0.080	1.022
A20	15	A20a	+ 15	n.d.	NEG	0.133	0.068	0.090	0.062	0.123
		A20b			1/128	0.226	0.354	1.312	0.094	1.418
A21	7	A21a	+ 14	n.d.	NEG	0.045	0.095	0.092	0.088	0.125
		A21b			1/32	0.041	0.189	0.109	2.952	2.938
A22	10	A22a	+ 13	n.d.	NEG	0.028	0.066	0.063	0.059	0.054
		A22b			1/152	0.229	0.332	0.157	0.092	0.229
A23	14	A23a		n.d.	1/32	0.096	0.088	0.166	0.077	0.126

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		A23b	+ 15		1/512	0.233	0.580	0.684	0.335	0.428
A24	8	A24a		n.d.	1/16	0.271	0.093	0.100	0.093	0.068
		A24b	+ 12		1/512	0.134	0.209	0.176	0.087	0.181
A25	10	A25a		n.d.	1/32	0.080	0.072	0.079	0.061	0.091
		A25b	+ 12		1/256	0.233	1.857	2.710	0.093	1.664
A26	15	A26a		n.d.	1/128	0.090	0.070	0.067	0.066	0.065
		A26b	+ 14		1/256	0.167	0.165	0.536	0.177	0.276
A27	7	A27a		n.d.	1/32	0.062	0.087	0.217	0.082	0.187
		A27b	+ 22		1/1024	0.406	1.599	2.849	2.007	1.752
A28	15	A28a		n.d.	NEG	0.095	0.075	0.080	0.076	0.070
		A28b	+ 19		1/512	0.185	1.544	0.317	0.078	0.244
A29	7	A29a		n.d.	1/32	0.065	0.127	0.114	0.108	0.089
		A29b	+ 11		1/256	0.160	1.139	0.546	0.085	0.838
A30	11	A30a		n.d.	NEG	0.048	0.091	0.095	0.087	0.068
		A30b	+ 16		1/1024	0.051	0.195	0.088	0.083	0.183
A31	9	A31a		n.d.	NEG	0.035	0.146	0.067	0.064	0.068
		A31b	+ 37		1/256	0.210	2.790	0.957	0.145	0.896
A32	16	A32a		n.d.	NEG	0.113	0.090	0.170	0.493	0.143
		A32b	+ 11		1/64	0.727	2.879	0.431	0.639	1.894
A33	8	A33a		n.d.	1/1024	0.109	0.320	0.540	1.672	0.308
		A33b	+ 17		1/256	0.229	0.358	2.192	2.906	1.521
A34	7	A34a		n.d.	<1/102	0.036	0.071	0.146	0.070	0.078
		A34b	+ 24		1/128	0.280	2.778	2.636	0.550	1.196
A35	12	A35a		n.d.	NEG	0.046	0.079	0.075	0.074	0.095
		A35b	+ 19		1/512	0.238	0.866	0.507	0.163	0.274
A36	14	A36a		n.d.	1/32	0.358	0.116	0.122	0.094	0.102
		A36b	+ 14		1/512	0.705	0.262	0.819	0.085	0.631
A37	39	A37a		+	1/1024	0.414	0.635	0.432	0.105	0.515
		A37b	+ 34		n.d.	0.233	0.266	1.182	0.083	0.643
A38	3	A38a		n.d.	1/256	0.089	0.413	0.358	0.247	0.318
		A38b	+ 28		1/512	0.448	3.081	2.953	0.182	2.114
A39	9	A39a		n.d.	NEG	0.034	0.104	0.097	0.089	0.051
		A39b	+ 14		1/64	0.091	0.239	0.188	2.895	2.339
A40	12	A40a		+	n.d.	0.142	0.082	0.093	0.114	0.085
		A40b	+ 28		n.d.	0.180	0.803	0.581	0.112	0.658
A41	28	A41a		+	n.d.	0.177	1.192	0.117	0.088	0.078
		A41b	+ 16		n.d.	0.656	3.075	0.141	0.505	1.873
A42	21	A42a		+	n.d.	0.156	0.071	0.093	0.066	0.079
		A42b	+ 30		n.d.	0.481	1.508	1.664	1.983	1.655
A43	14	A43a		+	n.d.	0.042	0.102	0.072	0.089	0.074
		A43b	+ 26		n.d.	0.170	0.760	0.560	0.098	0.607
A44	13	A44a		+	n.d.	0.207	0.290	0.172	3.208	3.165
		A44b	+ 13		n.d.	0.374	1.168	1.865	3.098	3.062
A45	12	A45a		+	n.d.	0.037	0.135	0.153	0.120	0.161
		A45b	+ 18		n.d.	0.060	0.316	0.332	0.336	0.284
A46	6	A46a		+	n.d.	0.036	0.070	0.204	0.072	0.189
		A46b	+ 19		n.d.	0.075	0.317	0.734	0.070	0.393
A47	10	A47a		+	n.d.	0.122	0.599	0.246	0.170	0.406
		A47b	+ 25		n.d.	0.207	0.646	0.557	0.179	0.470

^a PCR, Polymerase Chain Reaction on sputum and/or pharyngeal swab.

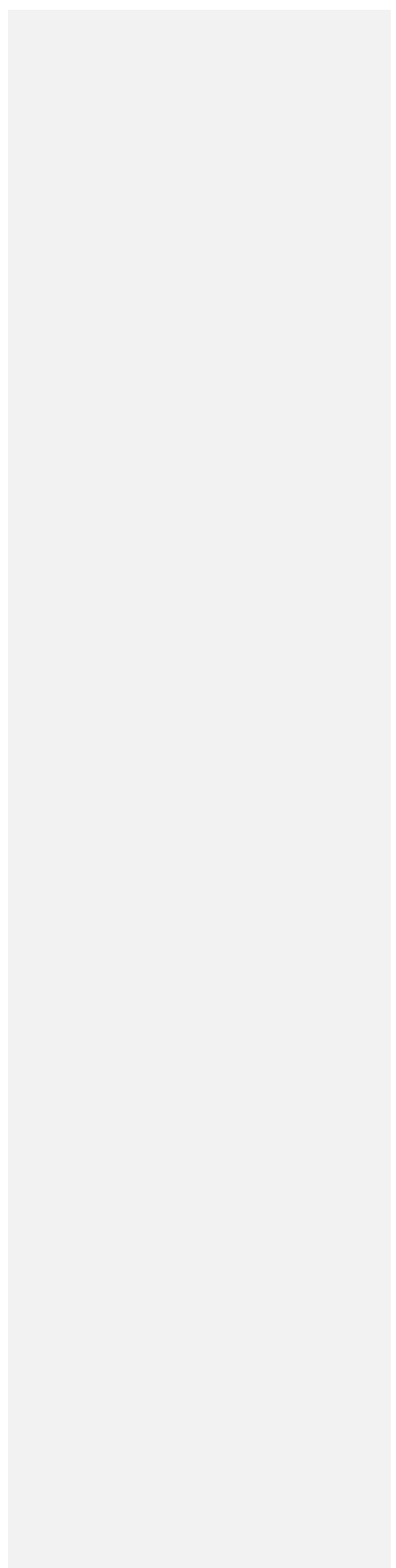
^b CF, Complement Fixation test expressed in serum dilution.

^c Cut off value for the commercial IgG ELISA was 0.358.

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^d Cut off values for the IgG ~~Rec-ELISAs~~Rec-ELISA's were 0.232 (GST-P1), 0.268 (GST-P30), 0.209 (GST-MPN456), 0.219 (GST-EC12).

Bold-face type, value greater than cut off; n.d., not determined; NEG, negative.



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4 **Table 2**

5
6 Diagnostic performance of the IgG ELISAs with sera from *M. pneumoniae* infected
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8 individuals
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IgG assay	No. of positive diagnosis^a	Sensitivity (%)	Specificity (%)	PPV^b (%)	NPV^b (%)	<i>p</i>^c
GST-P1	41	87.2	100	100	66.7	< 0.001
GST-P30	38	80.9	100	100	57.1	< 0.001
GST-MP456	19	40.4	100	100	30.0	0.005
GST-EC12	44	93.6	100	100	80.0	< 0.001
IBL assay	17	36.2	100	100	27.3	0.125

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26 ^aPatients with at least one positive result by analysing paired sera.

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28 ^bPPV, positive predictive value; NPV, negative predictive value.

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30 ^c*p* values were determined by two-tailed Fisher exact test by comparison of the results for
31 sera from patients with *M. pneumoniae* infection (*n*=47) and sera from patients with
32 pneumococcal pneumonia (*n*=12)
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Table 3

Immunoreactivity of recombinant antigens with sera from patients with a suspected *M. pneumoniae* infection (group B)

Patient	Age (years)	Serum sample	Days from first sample	IgG CF ^a	IgG ELISA ^b	IgG Rec-ELISA ^c			
						GST-P1	GST-P30	GST-MP456	GST-EC12
B1	4	B1a	+ 12	n.d.	0.040	0.058	0.062	0.100	0.069
		B1b			0.041	0.062	0.063	0.098	0.071
B2	5	B2a	+ 10	NEG	0.050	0.062	0.107	0.056	0.220
		B2b			0.055	0.089	0.114	0.070	0.277
B3	4	B3a	+ 12	NEG	0.092	0.103	0.111	0.091	0.081
		B3b			0.066	0.104	0.097	0.067	0.068
B4	3	B4a	+ 14	n.d.	0.053	0.091	0.091	0.084	0.078
		B4b			0.049	0.063	0.066	0.121	0.058
B5	6	B5a	+ 16	NEG	0.041	0.086	0.075	0.057	0.052
		B5b			0.092	0.071	0.081	0.033	0.066
B6	4	B6a	+ 11	NEG	0.046	0.135	0.092	0.090	0.157
		B6b			0.039	0.097	0.061	0.051	0.082
B7	4	B7a	+ 15	NEG	0.044	0.057	0.056	0.051	0.052
		B7b			0.079	0.057	0.050	0.051	0.047
B8	2.3	B8a	+ 30	n.d.	0.046	0.055	0.052	0.059	0.047
		B8b			0.048	0.049	0.046	0.049	0.052
B9	2	B9a	+ 16	NEG	0.041	0.081	0.069	0.066	0.069
		B9b			0.041	0.068	0.057	0.066	0.058
B10	1.7	B10a	+ 26	n.d.	0.039	0.062	0.058	0.062	0.206
		B10b			0.065	0.076	0.066	0.065	0.168
B11	8	B11a	+ 11	NEG	0.050	0.059	0.072	0.042	0.063
		B11b			0.075	0.073	0.084	0.053	0.062
B12	3	B12a	+ 18	n.d.	0.041	0.113	0.080	0.063	0.091
		B12b			0.036	0.117	0.097	0.063	0.071
B13	3	B13a	+ 31	n.d.	0.038	0.112	0.086	0.067	0.077
		B13b			0.039	0.102	0.086	0.062	0.063
B14	5	B14a	+ 15	n.d.	0.043	0.089	0.081	0.087	0.064
		B14b			0.041	0.107	0.077	0.080	0.067
B15	4	B15a	+ 12	n.d.	0.159	0.123	0.117	0.119	0.108
		B15b			0.045	0.103	0.088	0.072	0.082
B16	5	B16a	+ 9	n.d.	0.039	0.079	0.070	0.068	0.066
		B16b			0.035	0.067	0.064	0.059	0.063
B17	23	B17a	+ 17	n.d.	0.077	0.058	0.084	0.107	0.132
		B17b			0.056	0.055	0.074	0.086	0.112
B18	1.6	B18a	+ 8	n.d.	0.037	0.097	0.079	0.091	0.104
		B18b			0.045	0.086	0.074	0.069	0.087
B19	2	B19a	+ 12	n.d.	0.054	0.068	0.063	0.065	0.057
		B19b			0.038	0.084	0.066	0.059	0.059
B20	5	B20a	+ 34	n.d.	0.034	0.097	0.077	0.061	0.060
		B20b			0.037	0.093	0.072	0.067	0.077
B21	2	B21a	+ 13	n.d.	0.063	0.118	0.069	0.064	0.072
		B21b			0.041	0.133	0.066	0.102	0.111
B22	5	B22a	+ 24	n.d.	0.048	0.089	0.072	0.078	0.064
		B22b			0.037	0.059	0.064	0.069	0.066
B23	3	B23a	+ 10	n.d.	0.031	0.065	0.071	0.070	0.064
		B23b			0.039	0.078	0.083	0.050	0.062

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B24	9	B24a		n.d.	0.067	0.069	0.066	0.052	0.069
		B24b	+ 16		0.039	0.070	0.063	0.065	0.058
B25	1.6	B25a		n.d.	0.034	0.113	0.115	0.100	0.075
		B25b	+ 11		0.039	0.114	0.078	0.077	0.067
B26	4	B26a		n.d.	0.036	0.083	0.063	0.082	0.069
		B26b	+ 10		0.035	0.075	0.062	0.056	0.060
B27	2.5	B27a		n.d.	0.161	0.106	0.077	0.066	0.078
		B27b	+ 15		0.075	0.125	0.081	0.061	0.092
B28	18	B28a		NEG	0.109	0.086	0.157	0.084	0.318
		B28b	+ 23		0.142	0.083	0.152	0.083	0.290
B29	43	B29a		NEG	0.069	0.057	0.303	0.063	0.491
		B29b	+ 15		0.075	0.059	0.260	0.058	0.511
B30	4	B30a		n.d.	0.045	0.072	0.073	1.289	2.425
		B30b	+ 18		0.040	0.064	0.064	1.515	2.740
B31	10	B31a		n.d.	0.204	3.482	1.980	0.063	3.161
		B31b	+ 15		0.202	3.342	1.642	0.065	3.148
B32	25	B32a		NEG	0.048	0.083	0.111	0.066	0.096
		B32b	+ 17		0.117	1.548	0.269	0.044	2.940
B33	7	B33a		NEG	0.241	1.334	2.502	0.128	3.064
		B33b	+ 20		0.184	2.451	2.756	0.079	3.138
B34	8	B34a		NEG	0.146	2.285	0.221	0.134	2.792
		B34b	+ 18		0.200	3.000	1.976	0.504	3.149
B35	5	B35a		n.d.	0.051	0.292	0.241	0.375	0.584
		B35b	+ 19		0.103	1.699	1.534	0.448	2.993
B36	11	B36a		n.d.	0.037	0.072	0.069	0.066	0.052
		B36b	+ 7		0.114	0.526	0.255	0.073	0.720
B37	6	B37a		n.d.	0.035	0.059	0.055	0.140	0.206
		B37b	+ 17		0.260	2.866	0.845	0.226	3.086
B38	9	B38a		n.d.	0.059	0.105	0.113	0.085	0.518
		B38b	+ 14		0.092	0.921	0.380	0.135	2.708
B39	1.5	B39a		n.d.	0.120	0.367	0.366	0.059	1.491
		B39b	+ 13		0.160	0.511	1.083	0.060	2.944

^a CF. Complement Fixation test.

^b Cut off value for the commercial IgG ELISA was 0.358.

^c Cut off values for IgG [Ree-ELISAs](#)/[Rec-ELISA's](#) were 0.232 (GST-P1). 0.268 (GST-P30). 0.209 (GST-MPN456). 0.219 (IgG EC12)

Bold-face type. value greater than cut off; n.d.. not determined; NEG. negative.

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Figure 1

Schematic representation of the chimeric antigen GST-EC12

