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ORIGINAL ARTICLE

Antibody to *Chlamydia trachomatis* proteins, TroA and HtrA, as a biomarker for *Chlamydia trachomatis* infection

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Abstract We studied whether antibody to two chlamydial proteins (TroA and HtrA) could be used as biomarkers of *Chlamydia trachomatis* infection. Methods: Recombinant proteins *C. trachomatis* TroA and HtrA were used as antigens in enzyme immunoassay (EIA). Both IgG and IgA antibody responses were studied. Results: IgG or IgA antibody to either protein was infrequently detected in sera from healthy blood donors or virgin girls. Patients attending the STI Clinic and patients with perihepatitis had often IgG antibody against TroA (25 and 50 % respectively) and HtrA (21 and 38 % respectively). Especially in sera from patients with chlamydial perihepatitis, the A_{450nm} values with TroA were high (mean 1.591). A positive correlation between *C. trachomatis* MIF antibody and TroA ($r = 0.7$) as well as HtrA ($r = 0.5$) antibody was observed in sera from STI clinic patients and perihepatitis patients. Individuals with *C. trachomatis* infection and positive serology already when seeking medical attention had higher A_{450nm} values for TroA (0.638) and HtrA (0.836) than patients with no marker of previous exposure or with no infection (0.208 and 0.234 respectively). Diagnosis of genital *C. trachomatis* infection is often NAAT-based, whereas serology has little value in testing for uncomplicated genital *C. trachomatis* infection. TroA and HtrA antibodies are potential biomarkers for evaluation of ascending and repeated *C. trachomatis* infection.

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Introduction

Chlamydia trachomatis, an obligate intracellular bacterium, causes one of the most common sexually transmitted infections worldwide. The clinical spectrum varies from acute asymptomatic infection to symptomatic infection including endometritis, salpingitis, peritonitis, and perihepatitis, and even prolonged or persistent infection with severe sequelae, such as infertility, ectopic pregnancy, and chronic pelvic pain [1]. *C. trachomatis* is characterized by its unique, biphasic developmental cycle: it alternates between forms termed *elementary body* (EB) which is the infectious, extracellular form, and *reticulate body* (RB) which is the replicative, intracellular form. Persistent chlamydial infection is defined as a stage where viable chlamydial particles are present in cells but they cannot be cultivated [2]. In vitro, persistent infection can be induced by several factors [3, 4], such as amino acid starvation, viral co-infection [5], INF-γ [2], exposure to penicillin [6], and iron deprivation [7, 8].

Laboratory diagnosis of urogenital *C. trachomatis* infection often relies on nucleic acid amplification tests (NAAT), having high sensitivity and specificity [9]. The specimen material needs to contain chlamydial DNA, but not necessarily infected cells or live bacteria, although *C. trachomatis* is an intracellular microbe. In cases of persistent infection or infection in deeper tissues, sampling would require invasive specimen or biopsy collection from the infected tissues. Therefore, a serum-based method would be optimal to complement evaluation of *C. trachomatis*-related conditions. Micro-immunofluorescence serology (MIF), which uses purified bacteria as antigen, is considered the gold standard in serological diagnosis of *C. trachomatis*, but the obtained IgG titres as such do not distinguish past immunity from persistent infection. Studies of the transcriptional and proteomic patterns of acute and persistent chlamydial infections in vitro have

demonstrated that some proteins indeed are differentially expressed during persistence [10–13], and such proteins can be of diagnostic value [14, 15].

We evaluated two differentially expressed proteins, TroA and HtrA, as antigens in EIA. TroA (YtgA), encoded by *CT067*, is a substrate binding protein of the chlamydial iron-transport system (ABC transporter). TroA is present in infected cells but in smaller amounts in *C. trachomatis* EBs [16]. The amount of TroA increased when *C. trachomatis* was cultured under iron-starvation conditions, which is one means to induce persistence in vitro [17]. High temperature requirement protein (HtrA), encoded by *CT823*, plays a role during *C. trachomatis* replication [18]. HtrA levels increased later during the developmental cycle when *C. trachomatis* was cultured at the presence of penicillin, another means to induce persistence in vitro [19]. Use of such differentially expressed antigens in an easy-to-use format might offer a means to develop chlamydial serodiagnostics. We wanted to determine whether antibodies to TroA and HtrA could be used as markers of chlamydial infection. *C. trachomatis* TroA and HtrA proteins were cloned into *E. coli* and the recombinant proteins were used as antigens in EIA. Antibody responses were studied in serum specimens from individuals with known infection status [5, 20–22].

Materials and methods

Serum samples

Human serum specimens used in this study were panels from our previous projects, and included: (i) 111 sera from 72 women with symptoms of perihepatitis (40 of them had been considered as having chlamydial perihepatitis based on serology [22], (ii) 156 sera from patients (76 *C. trachomatis* NAAT-negative, 80 *C. trachomatis* NAAT-positive or culture-positive) attending the STI Clinic of Helsinki University Hospital, Finland, due to suspected *C. trachomatis* infection [5, 21], (iii) 37 sera from healthy, randomly collected, anonymous blood donors, and (iv) 48 serum samples from sexually inexperienced girls (mean age 12 yrs, range 8–17) [20]. To determine the possible cross-reactivity with *C. pneumoniae* antibodies, we studied a panel consisting of 45 sera from patients with acute *C. pneumoniae* infection proven with the classical micro-IF method (MIF).

Expression and purification of *C. trachomatis* TroA and HtrA

The genes encoding TroA and HtrA were amplified from *C. trachomatis* serovar D (purified DNA from strain UW-3/Cx ATCC VR-885 was used as a template). Primers to amplify the TroA (*CT067*) included EcoRI and XhoI recognition sequences. Primers to amplify HtrA (*CT823*) included BamHI and NotI recognition sequences. The PCR products were purified (Qiagen QIAquick PCR purification kit) and digested with the corresponding restriction enzymes, cloned in the expression vector pGEX-4 T-3 in frame with the GST fusion tag, and transformed into *E. coli* DH5α-strain. A 250–300 ml culture of the bacterial cells were grown in LB +37 °C until OD600 > 0.5 and then induced for 5–6 hours with 0.5 mM IPTG (Promega) at +30 °C to express the recombinant proteins. Cells were harvested by centrifugation for 15 minutes at 6,000 × g, suspended to 15–20 ml of PBS and lysed by sonication (4 × 10 sec, with 50 % amplitude), after which the lysate was cleared by centrifugation for 10 minutes at 5,000 rpm. The expressed proteins were purified from the cleared lysate by Glutathione Sepharose 4B (GE Healthcare) according to manufacturer's instructions for batch purification. Briefly, 250 µl of 50 % sepharose was added per 10 ml of cleared lysate, and incubated overnight on a shaker at +4 °C. After three washes with PBS, sepharose-bound TroA and HtrA were eluted by 10 mM and 50 mM glutathione respectively. The protein concentration (A_{280}) of each eluate was measured by NanoDrop, and the purity was evaluated by SDS-PAGE and western blotting (Fig. 1).

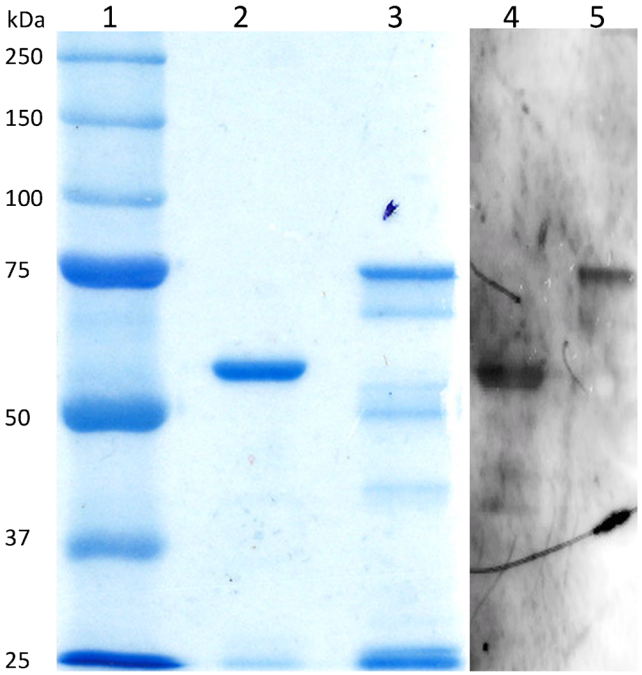

Fig. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (*lanes 2–3*) and western analysis (*lanes 4–5*) of purified recombinant GST-tagged *C. trachomatis* TroA and HtrA proteins following elution from Glutathione Sepharose 4B with glutathione. PrecisionPlus Protein™ molecular weight markers (Bio-Rad) (*lane 1*). A patient serum was used to detect the proteins in western blot

Enzyme immunoassays

Purified CT067 and CT823 proteins were diluted in 0.05 M carbonate–bicarbonate buffer (Sigma–Aldrich), and 100 ng and 400 ng of the protein was coated to Nunc Maxisorp ELISA plate wells for IgG and IgA ELISA respectively. After coating for o/n at +4 °C, the wells were washed 5 times with 1XPBS + 0.05 % Tween, and blocked at +37 °C with 3 %-BSA-1xPBS for 1 h. Wells were again washed 5 times with PBST, and 100 μl of sera diluted 1:200 for IgG-EIA and 1:50 for IgA-EIA in Sample dilution buffer (Labsystems Diagnostics) were applied to ELISA plate, and incubated on a shaker for 1 h, at room temperature. After 5 washes with PBST, 100 μl of the 1:1,000 diluted polyclonal anti-Human IgG-HRP (Dako) or 1:2000 diluted polyclonal anti-human IgA-HRP (Dako) conjugate was added, and incubated again on a shaker for 1 hour at room temperature. Finally, the plates were again washed 5 times with PBST, and 100 μl/well of 3,3',5,5' - tetramethylbenzidine substrate (TMB, Labsystems Diagnostics, Vantaa, Finland) was added for 10 minutes. Reactions were stopped by adding 100 μl of 0.5 N H_2SO_4, and the A_{450nm} was measured. Each serum was analyzed in duplicate in antigen-coated and innon-coated wells, and the absorbance value of the non-coated well was subtracted from the absorbance value of the antigen-coated well. A positive serum (serum reactive with the purified protein in western blotting) and a negative serum as well as buffer-only wells were included in each run.

Both IgG and IgA antibody response in human sera with the recombinant TroA and HtrA protein were studied. The cut-off values were based on the absorbance values (mean + 2 SD) obtained using specimens of sexually inexperienced girls, not exposed to *C. trachomatis*, and was set as A_{450nm} 0.5 for TroA and HtrA IgG and A_{450nm} 0.4 for TroA IgA. In our preliminary experiments, IgA A_{450nm} values were extremely low when HtrA was used as antigen, and therefore only IgG responses were studied. The intra-assay coefficient of variation (CV) of the TroA and HtrA assays was 4.4–5.2 % and 4.9 % respectively. The inter-assay CV of the TroA assays was 11.6 % for IgG and 16.7 % for IgA. The inter-assay CV was 10.4 % for the HtrA assay.

To analyze specificity of the assays, sera from 45 patients with serologically verified acute *C. pneumoniae* infection were examined for reactivity against *C. trachomatis* TroA and HtrA. A very weak correlation was observed between *C. pneumoniae* IgG antibody (MIF) and *C. trachomatis* TroA or HtrA IgG antibody (the Spearman correlation coefficient for the data is and −0.094 and 0.206 respectively, both NS) (data not shown).

MIF serology

MIF serology was performed using purified elementary bodies of *C. trachomatis* as antigen [23]. Specific antibodies were detected using Anti-Human IgG (γ-chain specific) –FITC antibody produced in goat (Sigma). Antibody titres ≥32 were considered positive, and titres ≥128 high.

Statistical analysis

To assess inter-assay and intra-assay variation, the coefficients of variation (CV%) were calculated. Inter-assay performance was calculated using the net A_{450nm} values given by the control sera in eight to 11 independent experiments. The intra-assay CV was calculated as the mean CV of the A_{450nm} given by duplicate analysis of 45 samples. Mann–Whitney test *U* test was used to analyze differences in antibody measurements between the patient groups (KyPlot 5.0 data analysis and visualization software). Correlations between titres obtained by MIF and ranked EIA absorbance values were evaluated by Spearman's correlation coefficient (SPSS). Correlations were considered significant at levels ≤0.01.

Results

TroA as EIA antigen

IgG antibodies against TroA were common in sera from patients with perihepatitis (50.0 %), and in those attending the STI Clinic (25.0 %). As expected, antibody was more rarely detected in sera from healthy blood donors (8.1 %) and virgin girls (4.2 %). Most patients (82.5 %) with chlamydial perihepatitis (an ascending infection) and 23.8 % of those with chlamydial urogenital infection had TroA IgG antibody. The absorbance values were highest in the chlamydial perihepatitis group (mean A_{450nm} 1.591) and lowest in blood donors (mean A_{450} 0.216) and virgin girls (mean A_{450nm} 0.123) (Table 1).

Throughout this study, IgA antibody was rather infrequently detected. TroA IgA antibody was most often detected among women with chlamydial perihepatitis (22.5 %; $p < 0.05$ when compared to women with non-chlamydial perihepatitis). Overall, the A_{450nm} values for IgA were quite low (even in the chlamydial perihepatitis group the mean A_{450nm} was 0.241, ranging from negative to 0.796).

Sera from STI Clinic patients and from patients with perihepatitis were also studied by Micro-IF. Reactivity of these sera in MIF and with recombinant TroA protein is shown in Fig. 2a. Of the sera with high MIF titres (≥128) 75 % were positive by TroA EIA. The correlation coefficient for the MIF and TroA IgG data was 0.650 (for NAAT-positive

Table 1 Prevalence of antibody against *C. trachomatis* TroA and HtrA and mean A_{450nm} value in the patient groups and controls

	Virgin girls	Blood donors	STI clinic clients		Perihepatitis patients	
			Ctr neg	*Ctr* pos	*Ctr* MIF ≥128	*Ctr* MIF ≤64
N	48	37	76	80	40	32
TroA IgG						
Positive (%)	2 (4.2)	3 (8.1)	20 (26.3)	19 (23.8)	33 (82.5)	3 (9.4)
Mean A_{450nm}	0.123	0.216	0.412*	0.374*	1.591***	0.295
TroA IgA						
Positive (%)	4 (8.3)	3 (8.1)	10 (13.2)	7 (8.8)	9 (22.5)	0 (0)
Mean A_{450nm}	0.098	0.159	0.163	0.159	0.241**a	0.029
HtrA IgG						
Positive	1 (2.1)	2 (5.4)	10 (13.2)	23 (28.8)	25 (62.5)	2 (6.3)
Mean A_{450nm}	0.097	0.113	0.353	0.464**	0.979***	0.165
MIF IgG						
Positive %	0	NA	40.8 (31/76)	68.6 (35/51)	100#	21.9
GMT			18.7	48.1***	470***	11.6

The groups were compared with the nonparametric Mann–Whitney *U* test. Probabilities ≤0.05 (*), ≤0.01 (**) and ≤0.001 (***) are indicated in the table

* this group had significantly higher mean A_{450nm} compared to blood donors ($p \leq 0.05$)

** this group had significantly higher mean A_{450nm} compared to blood donors ($p \leq 0.01$)

**a this group had significantly higher mean A_{450nm} compared to perihepatitis patients with MIF ≤64 ($p \leq 0.01$)

*** this group had significantly higher mean A_{450nm} /GMT compared to any other group ($p \leq 0.001$)

by definition

0.559 and for perihepatitis 0.808; all correlations significant at the level 0.01). Patients attending the STI Clinic with suspected *C. trachomatis* infection were classified based on their NAAT status and MIF serology (Table 2).

Of the NAAT-positive patients with MIF titres ≥128 (suggesting previous and/or repeated infections), 55 % had TroA IgG antibody, while 96.8 % of sera from NAAT-positive individuals with low MIF titres (≤64; suggesting first urogenital infection) remained negative by TroA EIA. Moreover, individuals with *C. trachomatis* infection and positive serology already when seeking medical attention had higher A_{450nm} values for TroA (0.638) than MIF-negative patients (0.215; $p \leq 0.001$).

HtrA as EIA antigen

IgG antibodies against HtrA were most prevalent in sera from patients with chlamydial perihepatitis (62.5 %) and those with NAAT or culture-proven *C. trachomatis* urogenital infection (28.8 %). Among virgin girls and healthy blood donors, IgG antibodies against HtrA were detected in 2.1 and 5.4 % respectively (Table 1). Individuals with *C. trachomatis* infection and positive serology already when seeking medical attention had higher A_{450nm} values for HtrA (0.836) than MIF-negative patients (0.235, $p \leq 0.001$) (Table 2). The absorbance values were highest among patients with chlamydial perihepatitis (mean A_{450nm} 0.979). The mean absorbances were very low among virgin girls and blood donors (0.097 and 0.113 respectively) (Table 1).

Comparison of the reactivity against HtrA with titres obtained by MIF showed that 60 % of NAAT-positive individuals with MIF titres ≥128 (suggesting previous exposure) had IgG antibodies against HtrA antigen (Table 2). Meanwhile, 90.3 % of NAAT-positive individuals with low MIF titres (≤64, suggesting first urogenital infection) remained negative in HtrA-EIA. The correlation coefficient for the MIF and HtrA IgG data was 0.529 (for NAAT-positive 0.516 and for perihepatitis 0.578; all correlations significant at the level 0.01).

Kinetics of antibody response

Follow-up serum specimens were available from 18 individuals with chlamydial perihepatitis. Kinetics of TroA and HtrA antibody response was evaluated using sera from individuals with positive findings in any serum specimen (Fig. 3). This analysis shows that A_{450nm} for TroA and HtrA IgG antibody (Fig. 3a and b), remained detectable and rather stable for months after chlamydial perihepatitis, an infection ascending from the genital tract. The number of individuals with positive TroA IgA was limited (Fig. 3c), but analysis suggests that TroA IgA antibody showed a tendency to disappear faster than IgG antibody.

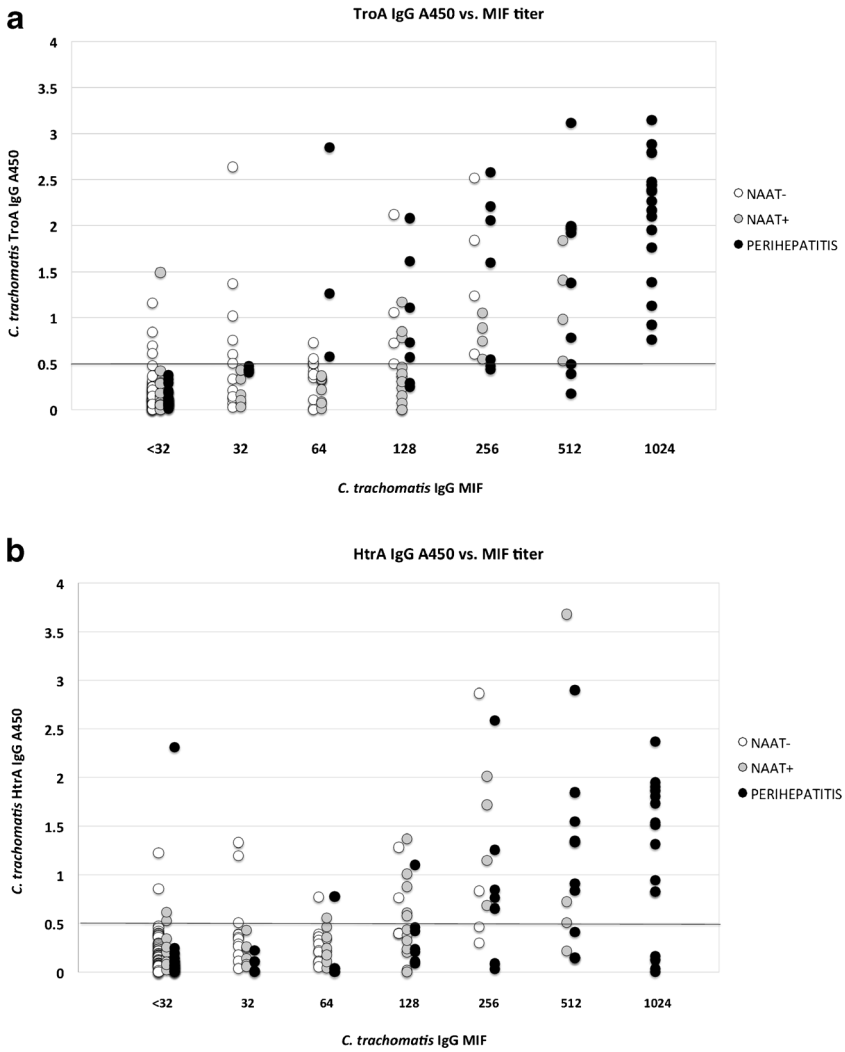

Fig. 2 **a** Correlation between serum TroA IgG antibody levels and *C. trachomatis* MIF IgG titres. Sera from individuals that were *C. trachomatis* NAAT positive (*gray circles*), NAAT negative (*white circles*) and had perihepatitis (*black circles*) are plotted according to their TroA IgG antibody level measured by A_{450nm} and their *C. trachomatis* IgG MIF titre. The TroA test cutoff used for was 0.5, marked by the line. Correlation is strong and significant at the 0.01 level (r = 0.650). **b** Correlation between serum HtrA IgG antibody levels and *C. trachomatis* MIF titres. Sera from individuals that were *C. trachomatis* NAAT positive (*gray circles*), NAAT negative (*white circles*) and had perihepatitis (*black circles*) are plotted according to their HtrA IgG antibody level measured by A_{450nm} and their *C. trachomatis* IgG MIF titres. The HtrA test cutoff used for was 0.5, marked by the *line*. Correlation is moderate and significant at the 0.01 level ($r = 0.529$)

Table 2 Prevalence of antibody against *C. trachomatis* TroA and HtrA and mean A_{450nm} value in STI clinic patients with suspected *C. trachomatis* infection

	STI clinic clients			
	"repeated infection"	"past infection"	"likely first infection"	"no infection"
MIF IgG serology	≥128	≥128	≤64	≤64
NAAT	*Ctr* pos	*Ctr* neg	*Ctr* pos	*Ctr* neg
N	20	8	31	68
TroA IgG				
Positive (%)	11 (55.0)	7 (87.5)	1 (3.2)	13 (19.1)
Mean A_{450nm}	0.638***	1.324***	0.215	0.310
HtrA IgG				
Positive	12 (60.0)	4 (50.0)	3 (9.7)	6 (8.8)
Mean A_{450nm}	0.836***	0.912**	0.235	0.282

The patients were classified according their MIF serology and NAAT results. The groups were compared with the nonparametric Mann–Whitney *U* test. Probabilities ≤0.01 (**) and ≤0.001 (***) are indicated in the table

** statistically significant difference between this group and the MIF ≤64 groups ($p \leq 0.01$)

*** statistically significant difference between this group and the MIF ≤64 groups ($p \leq 0.01$)

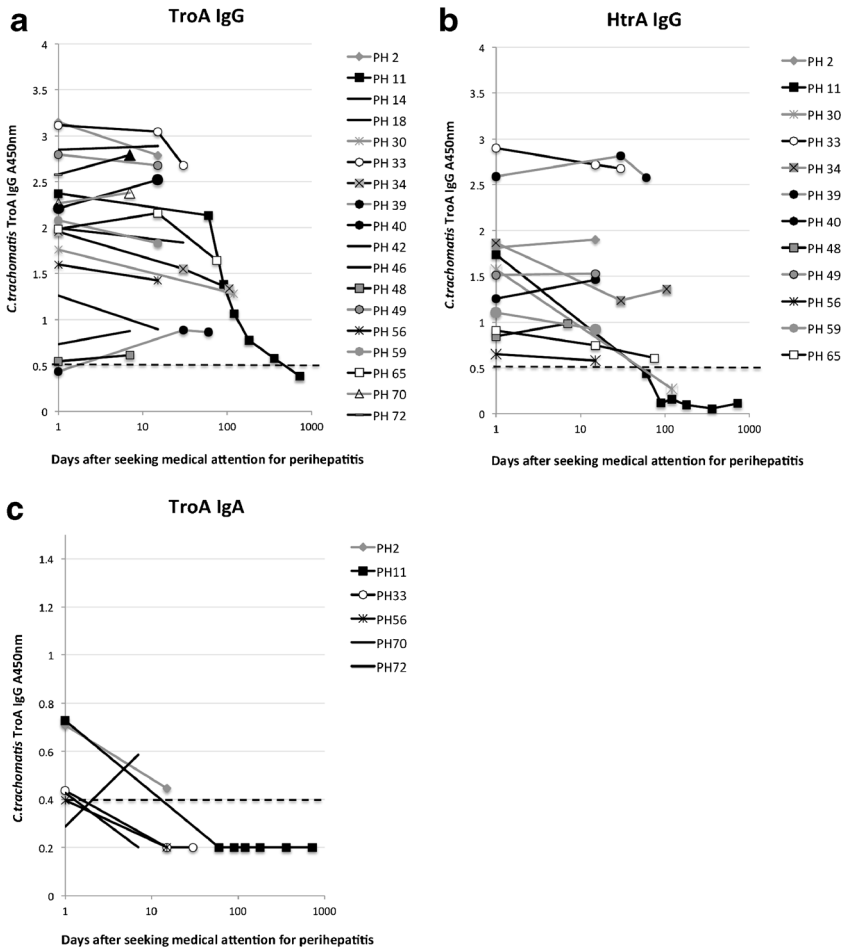

Fig. 3 Kinetics of antibody response in chlamydial perihepatitis. Sera from antibody positive individuals are plotted according to their TroA IgG (*a*), HtrA IgG (*b*) and TroA IgA (*c*) antibody level measured by A_{450nm} and time (days after seeking medical attention for perihepatitis). The *dashed line* marks the test cutoff used

Discussion

Novel EIAs for detection of antibody against two *C. trachomatis* encoded proteins are described and evaluated. It is shown that *C. trachomatis*-infected patients generate antibodies against TroA and HtrA. TroA protein is present in infected cells, in the chlamydial inclusion, but not in cytosol [16], and especially in cells cultured under iron-starvation [17]. HtrA plays a role during *C. trachomatis* replication [18] and especially under penicillin exposure [19]. The role of HtrA in pathogenesis of *C. trachomatis* is not clear, but it has been shown to carry out both proteolytic (serine protease) and chaperone-like activities [24]. HtrA is localized in chlamydial inclusion and is also secreted in the cytosol of the infected cells [25]. As both TroA and HtrA proteins are present in infected cells and thus represent potential novel biomarkers of infection, we evaluated their applicability in serology using our characterized serum panels.

Antibody to TroA or HtrA protein was rather infrequently detected in sera from healthy blood donors or virgin girls. This suggests that in populations where present or past *C. trachomatis* infection is rare, antibody to TroA and HtrA was rare. Furthermore, sera from patients with acute *C. pneumoniae* infection only occasionally reacted with these proteins. This confirms the earlier observation that sera with high titre antibody to *C. pneumoniae* did not detect *C. trachomatis* TroA [16]. These findings also suggest that the corresponding *C. pneumoniae* protein (CPn0349) that shares 60 % homology with *C. trachomatis* TroA, is either not immunogenic or if immunogenic, the antibody does not recognize *C. trachomatis* TroA. The lack of correlation between *C. pneumoniae* MIF antibody and TroA antibody further supports species-specificity of the assay.

C. trachomatis NAAT-positive STI Clinic patients with *C. trachomatis* antibody (MIF IgG ≥128) and patients with chlamydial perihepatitis had more antibodies against TroA (55 and 83 % respectively) and HtrA (60 and 63 % respectively). This suggests that in ascending *C. trachomatis* infection (such as perihepatitis) and on repeated exposure (represented by patients with infection and positive serology already at admission), these proteins are presented to the immune system and can elicit antibody response. Especially, in patients with chlamydial perihepatitis, the A_{450nm} values with TroA were high, suggesting that TroA is a potent immunogen. Furthermore, 24 % of NAAT-positive patients had TroA IgG antibody. This finding is in agreement with earlier smaller studies, in

which patients with culture-proven *C. trachomatis* genital infection were shown to generate antibodies against TroA [11, 26]. In our material, antibody to HtrA was in general less common than antibody to TroA. Most frequently, HtrA antibody was detected in sera from patients with chlamydial perihepatitis (63 %) and STI Clinic patients with urogenital *C. trachomatis* infection (29 %). Our observations further corroborate an earlier smaller study, in which patients with genital tract infection had antibody against full-length HtrA protein [28]. Furthermore, in another study, only sera from patients with MIF- and culture-confirmed *C. trachomatis* infection had antibodies against HtrA [29]. Also in our study, 60 % of MIF- and NAAT-positive patients had HtrA antibody.

Persistently elevated serum IgG and IgA antibody are associated with chronic *C. pneumoniae* infection [27]. Thus, IgA responses were also examined here to determine if antibody of IgA class could serve as an indicator of infection. However, fewer individuals in each group had IgA responses than IgG responses against TroA. The mean absorbance values for IgA were lower (0.098–0.241) than mean absorbance values for IgG (0.123–1.591). Our data modestly suggests that TroA IgA antibody could disappear faster that IgG antibody, but the small number of specimens available limits the analysis. Studying a larger panel of sequential specimens from individuals that cleared the infection, as well as from patients with tubal factor infertility or reactive arthritis, could shed light on this.

Together, our findings and the earlier reports suggest that TroA and HtrA antibody responses appear following acute phase of *C. trachomatis* infection. The response was at its strongest and most frequently detected in ascending infection, such as perihepatitis, or if *C. trachomatis* MIF serology and NAAT results suggested earlier or repeated exposure. Whether the results of the TroA and HtrA antibody assays tend to become negative following treatment of acute urogenital infection or remain positive during persistent infection is not known. This is of importance, as several lines of evidence suggest that persistent chlamydial infections do occur. Electron microscopy has demonstrated aberrant bodies of *C. muridarum* in uterine horns of infected mice treated with amoxicillin, an antibiotic known to induce persistence in cultured cells [30]. Moreover, chlamydial DNA can frequently be detected in the absence of culturable bacteria in human ocular and urogenital *C. trachomatis* infections [31, 32]. Individuals with MIF antibody detectable already when seeking medical care were likely to have had previous or repeated infections. In this group, 64 and 57 % of those with high MIF titre (≥128) were positive for TroA and HtrA respectively. As *C. trachomatis* antibody seems to persist after infection [33, 34], differential reactivity with TroA and HtrA could be of diagnostic value. It would be of interest to elucidate whether certain genes of *C. trachomatis* are expressed in vivo during persistent infection. In-vitro transcriptome and proteome analyses have shown that *C. trachomatis* gene expression is altered during persistence in vitro. This suggests that it might be possible to find an antigenic marker indicative of persistent infection. Both TroA and HtrA are differentially transcribed, at least in in-vitro conditions, and could thus represent such biomarkers. Indeed, in peptide and protein array-based studies, presence of antibody against HtrA or a HtrA-derived peptide was associated with chlamydial tubal factor infertility [14, 35].

In summary, TroA and HtrA EIA can complement serological investigation of *C. trachomatis* infection. Whether they can be used as biomarkers of persistent infection clearly warrants further studies.

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Compliance with ethical standards

Funding The funders listed above had no role in study design, data collection, and interpretation, or in the decision to submit the work for publication.

Conflict of interest The authors declare that they have no conflict or interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments. For collection of specimens in the 1980s and 1990s, formal consent was not required. However, for analysis of anonymous specimens from our earlier studies (refs. [20–22]), research permits were obtained from institutional research committee (Helsinki University Hospital, Laboratory Division; HUSLAB §29 09.05.2012). Informed consent was obtained from individuals participating in the ChlamyTrans study (ref. 5) whose sera were included in this study.

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