Evidence for Chlamydia in Crohn's Disease

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Abstract

Chlamydia cause enteritis in young pigs and calves, and granulomas within intestinal lymphatics. Recently we called attention to the lymphangitis and lymphatic obstruction that occurs in Crohn's disease. We searched resected tissues from patients for evidence of chlamydia and sera for evidence of previous exposure to chlamydia. Immunohistochemistry and real-time PCR were employed to seek chlamydia in preserved tissues. In the IHC, antibody to *C. trachomatis* served as primary antibody; in the PCR, primers specific for *Chlamydiaceae* were employed. Commercial ELISA kits measured anti-chlamydia IgG and IgA against *C. trachomatis* antigen in sera derived from a population of patients different from that which yielded the tissue specimens. IHC revealed focal positive staining for chlamydia in tissues of 5 of 19 patients. Positive reacting cells occurred within dense inflammation, in sparsely scattered macrophages in the submucosa and subserosa. Tissues from 3 of 22 control subjects were positive. Real-time PCR done on ileal, colonic, and regional lymph node tissues revealed evidence of chlamydia in 3 of 33 patients. Serology for anti-chlamydia IgG revealed 2 positive values in 24 patients, while serology for anti-chlamydia IgA revealed 4 positives among the 24 patients, and 1 positive in the 15 controls. One patient and one control had both elevated IgG and IgA titers. The 4 patients with elevated IgA titers were from a single family of 6 with Crohn's disease, which had been previously described. Additional consideration needs to be given to the chlamydia species, including those of animal origin, which leave behind little evidence of their previous involvement.

Keywords: Crohn's disease; Inflammatory bowel disease; Chlamydia

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Introduction

Recently we studied the lymphatics in resection specimens from patients with Crohn's disease [1, 2]. Employing serial sections of granuloma-obstructed lymphatics or of lymphatics that were distended with lymphocytes, we showed physical continuity between the former and the latter [2]. Given the importance of this lymphangitis, we began to search for viral or bacterial antigens that might be responsible [3].

In 2013, we took new interest in a previously-described chlamydial enteritis of young pigs, one that shows its greatest effect in the distal ileum [4]. After oral inoculation, *Chlamydia suis* invade and damage villous epithelial cells, then induce inflammation in villous lamina propria, and finally enter and damage lymphatic endothelium, the latter accompanied by intra- and extra-lymphatic inflammation [5, 6]. One consequence of this localized intestinal infection is the production of granulomas that obstruct mucosal, mucosal sub, muscularis, and serosal lymphatics [4, 6]. The finding that *C. suis* replicates what occurs in Crohn's disease raises the question: Is there evidence that human patients with Crohn's disease harbor or have been exposed to *C. suis* or other Chlamydia?

C. suis is closely related *to Chlamydia trachomatis* [7, 8]. The thought that the latter might be responsible for Crohn's disease had been examined earlier. Serologic studies over the years yielded equivocal results, as many studies showing antibody as those failing to find any (**Table 1**).

Table 1 Serologic studies seeking chlamydia antibody in patients with Crohn's disease.

Author	Year	Country	Ab	Antigen	Test	N +	Total	%	Ctr+	Ctr Total	Ctr % +
Rodaniche et al [34]	1943	US		LGV	SN	0	4	0			
Swarbrick et al [35]	1979	England	IgG	C trachomatis	MicroFA	4	54	7.4	7	50	14
Taylor-Robbinson et al [36]	1979	England		C trachomatis	Micro FA	8	55	14.5	5	23	22
Schuller et al [37]	1979	Holland	IgG	C trachomatis	Micro FA	38	55	69	1	50	2
Mardh et al [38]	1980	Sweden	IgG	C trachomatis	IIF	83	107	78	38	50	76
Gump et al [39]	1981	England		LGV (L2)	IIF	36	60	60	34	60	57
Elliot et al [9]	1981	England		C trachomatis	Micro FA	0	62	0	3	160	2
Orda et al [10]	1990	Israel	IgG	C trachomatis	IIP	14	15	93	4	15	26
Orda et al [10]	1990	Israel	IgA	C trachomatis	IIP	5	15	33	1	15	6
McGarity et al [16]	1991	England	IgG	C trachomatis	ELISA	7	48	15	14	48	29

Elliot *et al* seemed to have closed the door on this issue when they could not culture chlamydia from the tissues of patients (N=14), could not demonstrate chlamydia in tissue sections by FA (N=5), and could show antibody responses in only 3 of 62 patients [9]. Orda on the other hand found IgG responses to *C. trachomatis* in 14 of 15 patients (93%) [10].

There having been no search specific for *C. suis* and some variability in the methods and results of antibody testing of patients with Crohn's disease, we examined tissues from patients by immunohistochemistry and real-time polymerase chain reaction (PCR), and sera from patients by ELISA.

Materials and Method

Formalin-fixed paraffin-embedded tissue blocks from surgically resected diseased ilea and proximal colons from 19 CD patients from France and the United States were examined. These were transverse full thickness cuts, 1–3 per subject. Lennard-Jones criteria [11]. and histopathology were used for establishing diagnosis. Cases for study were selected for transmural obstructed lymphatics, and granulomas. Patients were 7 men and 12 women; aged 18–46 years (mean age 26). Formalin-fixed tissues from control patients were examined as well, 15 men and 7 women (mean age 48 years). Control tissues were from patients that had undergone colectomy for adenocarcinoma, ulcerative colitis, indeterminate colitis, diverticulitis, mesenteric venous thrombosis or gunshot trauma. Duration of disease ranged from 1 month to 9 years from the time of diagnosis. This study was approved by the Institutional Review Board of the University of Connecticut.

Primary antibodies for immunohistochemistry were obtained from commercial sources; mouse monoclonal anti-*Chlamydia trachomatis* LPS antibody [EVH-1] was employed to label chlamydia (this antibody cross-reacts with LPS of other chlamydia), (Abcam, Cambridge, MA). Microsections of tissues 4-μm thick were placed on positively charged glass slides and deparaffinized in xylene (Allegiance Healthcare, McGaw Park, IL, USA), rehydrated in a graded series of ethanol and rinsed with distilled water. Immunohistochemistry was performed as previously described [12]. Antigen retrieval was performed by using proteinase K for 10 minutes at room temperature. This was followed by washing in phosphate-buffered saline

(Dako, Carpinteria, CA); then endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 10 min., followed by application of Animal Free Blocker (Vector, Burlingame, CA) for 13 minutes at room temperature. After another wash in Dako wash buffer, tissue sections were incubated with the primary antibody, at a dilution of 1:1500 for 60 min at room temperature. After a short wash in Dako wash buffer, tissue sections were incubated for 30 min with HRP-labeled polymer conjugated with secondary antibody (EnVisionTM+ Dual Link System-HRP, Dako). Following a final wash in Dako wash buffer, sections were developed with Nova Red (Vector) for 2 min. After washing in warm tap water, the slides were counterstained with hematoxylin, then dehydrated, cleared and coverslipped. Appropriate negative and isotype controls (omitting primary antibody) were run with each batch of 20–40 test sections. Positive controls were *C. suis*-infected pig intestine.

Twenty micron thick sections from the paraffin-embedded tissues of 33 CD patients (the 19 from the IHC study and an additional 14 from France and the US) and the same 22 controls were submitted for real-time PCR. Genomic DNA was extracted using the Gentra PureGene Kit for tissues (Qiagen, Valencia, CA) and quantified with the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientifc Inc., Waltham, MA, USA). Samples were screened for *Chlamydia* spp. on the AB 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using the 23S rRNA gene based *Chlamydiaceae* family specific method, which includes primers Ch23S-F (5'-CTGAAACCAGTAGCTTATAAGCGGT-3'), Ch23S-R (5'-ACCTCGCCGTTTAACTTAACTCC-3') and probe Ch23S-P (FAM-CTCATCATGCAAAAGGCACGCCG) [13]. Each run included 2 positive controls, 2 negative controls, and samples. Positive controls used were sections of *C. suis*-infected pig intestine. Negative controls were urine samples previously screened for *Chlamydia trachomatis* (CT), *Trichomonas vaginalis* (TV) and *Neisseria gonorrhoeae* (NG) using a test developed and validated at the Translational Research Laboratory at the Dartmouth-Hitchcock Medical Center (Lebanon, NH, USA) [14]. Negative samples were negative for CT and TV, and positive for NG. Both samples and controls were run in duplicate. PCR was performed using 2x primer/probe mix, 2x TaqMan GTXpress Master Mix (Applied Biosystems) and 12ng of DNA. Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The positive

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samples showed a cycle threshold of <38.00. Samples positive for *Chlamydia* spp. were confirmed by melt curve analysis using the SmartCycler II (Cepheid, Sunnyvale, CA). PCR was performed using 25x primers, 2x SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA) and 12ng of DNA. Cycling conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The melt curve was determined at 65-95°C, 0.2°C per 1 sec. Positive samples showed a specific melting temperature (Tm) of approximately 84°C.

Sera from a separate set of CD patients (N=24) and sex- and age-matched controls (N=15) from northern France were tested for anti-chlamydia antibodies. ELISA was performed using Anti-*Chlamydia trachomatis* IgG Human ELISA Kit, and Anti-*Chlamydia trachomatis* IgA Human ELISA Kit (Abcam, Cambridge, MA) according to the manufacturer's instructions. Briefly, all materials were equilibrated to room temperature prior to use. For initial detection of specific antibodies serum samples were diluted 1:100 with sample diluent as indicated in manufacturer's instructions. Limited cross-reactivity data are available for the kit used. Positive samples were titrated further. Diluted samples were added into wells in duplicates and incubated at 37°C for 1 hour. The wells were washed five times with 1X washing solution. After the last wash *Chlamydia trachomatis* anti-IgG HRP or anti-IgA HRP conjugate was added and incubated at room temperature for 30 minutes. The plates were washed as above and TMB substrate solution was added into each well and incubated at room temperature in the dark for 15 minutes, when a stop solution was added to the wells. The absorbance was measured at 450nm in a Biotek Synergy HT microplate reader (Biotek Winosski, VT). The kit manufacture's criteria were used to validate the test and score the samples as positive or negative.

Results

Immunohistochemistry revealed focal positive staining for chlamydia in the tissues of five of 19 patients with Crohn's disease. Positive reacting cells occurred within dense inflammation, in sparsely scattered macrophages in the submucosa and sub-serosa (**Figure 1**). Focal positive staining also occurred in 3 of 22 controls, one resection for ulcerative colitis, one for indeterminate colitis, and one for mesenteric thrombosis. Real-time PCR performed on DNA extracted from ileal, colonic and regional lymph node tissues revealed evidence of *Chlamydia* spp. in three of 33 patients tested, two ileums (**Figure 2**) and one lymph node, and in none of 22 controls.

ELISA serology for anti-chlamydia IgG revealed two positive values in sera of 24 CD patients tested versus one positive in 15 controls. ELISA serology for anti-chlamydia IgA revealed four positives in the 24 patients tested and one positive in the 15 controls. One patient, number 9, and one control, number 19, had both elevated IgG and IgA titers. Importantly, the four patients who had elevated chlamydia-specific IgA antibodies were from a family of 6, previously described, all of whom had CD [15]. Duplication of this testing, done with a second ELISA kit carrying a different lot of antigen, confirmed these findings. Further titration of the positive sera detected IgA antibody titers of 1/400 in one patient serum and 1/200 in another patient. A similar titer of 1/200 was detected in one control serum.

Discussion

There is no strong association here between the presence of *Chlamydia* spp. and Crohn's disease. McGarity *et al*, using a chlamydia plasma probe and PCR, found no evidence of *C. trachomatis* in 5 resection specimens and 5 biopsies from CD patients [16]. Employing a PCR-EIA method for the 16S rRNA gene of *C. trachomatis* on mucosal biopsies Chen *et al* found one positive colon in 11CD patients and one positive colon in 37 controls [17].

Why then should we care about five positives here, three positives there, and four positives in another population? These positive data are small in number; however, among the microbial agents known to induce a lymphangitis, there are few that localize to the intestine and induce granulomatous obstruction of lymphatics [5, 6].

One needs to recognize that by the time our specimens were acquired from patients, the disease had been present for many years. Experimental studies in which young pigs, calves, and non-human primates were inoculated with chlamydia have shown that the organisms cannot be demonstrated in the tissues after 12 weeks [5, 18]. The chlamydia are known to persist in tissues in a form that cannot be readily demonstrated [19-21]. These dormant forms can be reactivated. When *C. trachomatis* strains were inoculated into rectal mucosa in monkeys, the LGV strain generated lymphoid follicles and giant cells within six weeks [18]. Intracytoplasmic inclusions were seldom seen after three weeks. Inflammatory follicles that were created (tertiary lymphoid organs) were well-developed with germinal centers, and persisted [18], however immunohistochemistry for chlamydia was negative after 10-12 weeks.

The duration of antibodies to chlamydia is variable, and waning with time [18, 22, 23]. And, they only indicate exposure during some point in the lifetime of the subject. Pathogen-specific serum IgA has been recognized as an important indicator of a number of viral infections [24] (Epstein-Barr virus [25, 26], rubella [27], varicella-zoster [28], cytomegalovirus [29]). In chlamydial infections, elevated levels of serum IgA have been regarded as an indication of either deep-seated infection [30] or active chlamydial disease [24, 31].

That all four of our IgA positives were from one family with CD is remarkable. Did the family have point source contact with *C. trachomatis* of other organ systems? Were they exposed to a chlamydia other than *C. trachomatis* that cross-reacted serologically? Or does this finding reveal important information about the etiology of Crohn's disease? In 1990, and again in 1992, the medical records of each member of this family were extensively reviewed, and each of the living were interviewed in the presence of the family gastroenterologist [15]. At that time, the time the sera were acquired, there was no evidence of other chlamydia- associated disease, leaving us to conclude that the elevated serum IgA titers to chlamydia reported here are a reflection of intestinal disease. We do wish to acknowledge that in our testing there may have been cross-reactivity with chlamydia
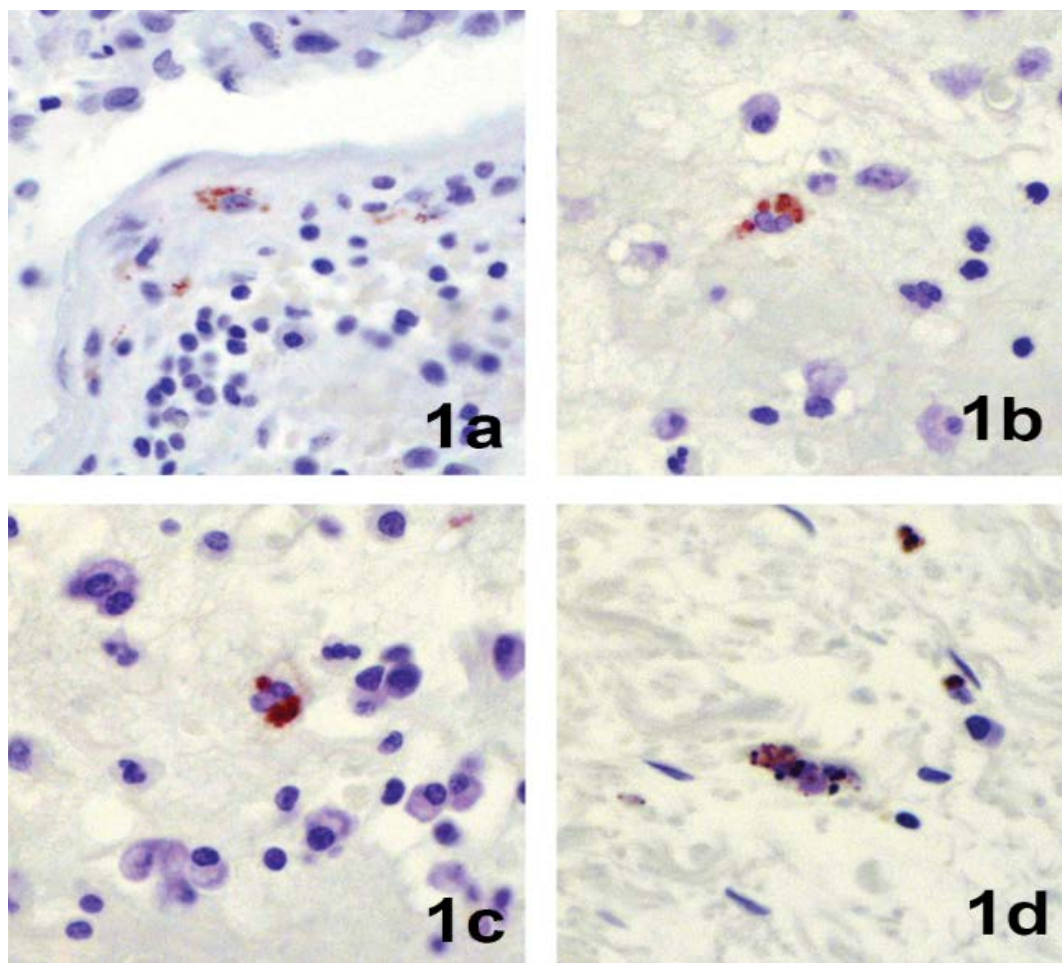
Figure 1 Positive immunoreactivity for chlamydia in macrophages located in regions of inflammation, 1a between colonic muscle coats and the serosa, adjacent to a fistula; 1b, 1c and 1d in ileal subserosa (1b and 1c from the same patient). (Original magnification: 400x).

other than *C. trachomatis*, perhaps chlamydia of animal origin. The real-time PCR was broadly specific for Chlamydiaceae 23SrRNA.

In the search for an etiologic agent in Crohn's disease, the chlamydia are sound candidates; some species induce lymphoid follicles, some induce granulomas, and some do both. Most importantly, however, some penetrate the intestinal mucosa and damage lymphatics, as in the pig disease [4-6] and in calves [22, 32, 33]. Additional consideration needs to be given to the *Chlamydia* spp. – however, defining the initiator of Crohn's disease may be impossible, given the unknown time from (a presumed) gastroenteritis in childhood or young adulthood to clinical manifestation of the effects of damaged ileal or ileocolonic lymphatics.

Acknowledgement

IRB Approval

This study was approved by the Institutional Review Board of the University of Connecticut.
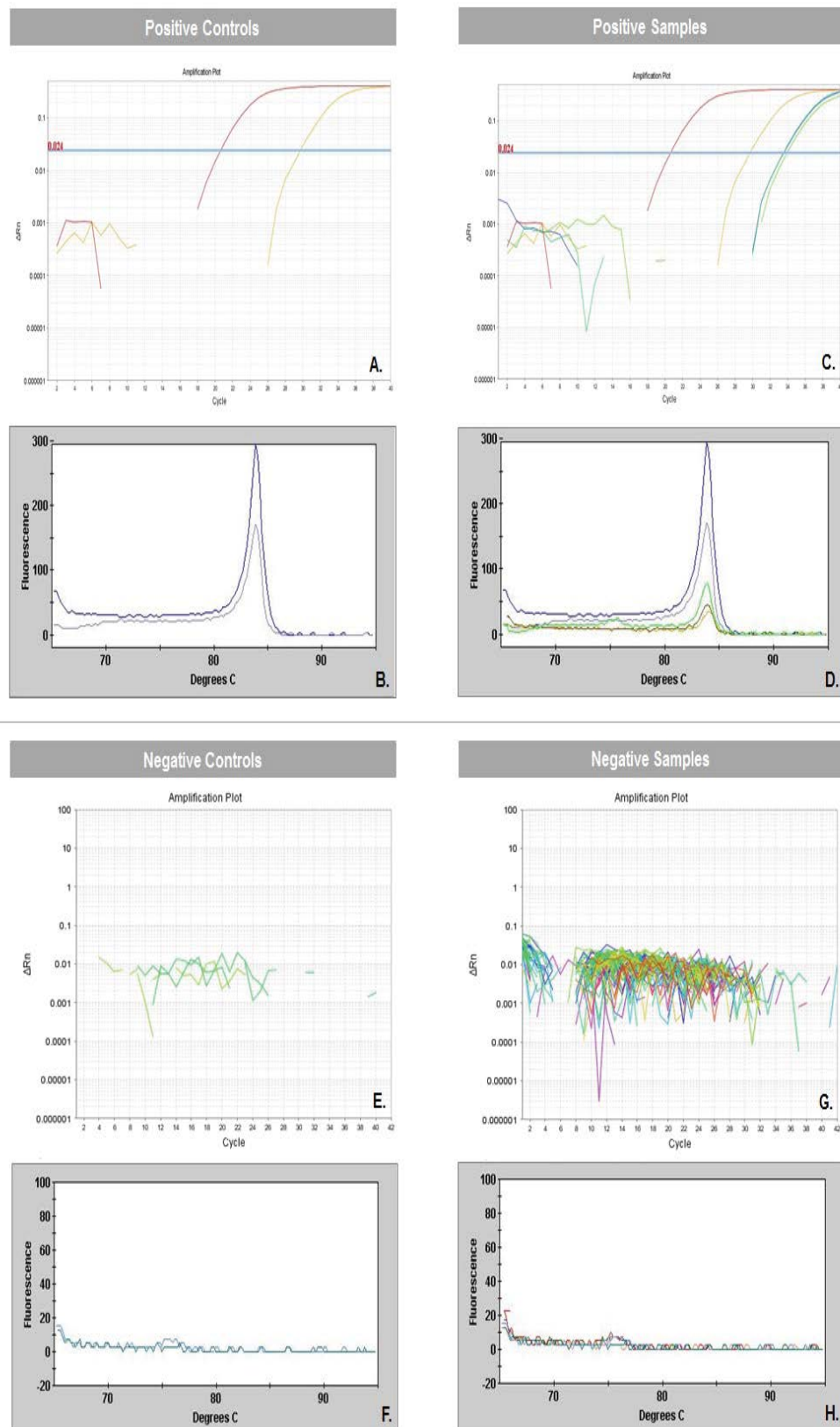
Figure 2: Real Time PCR results on both AB 7500 Fast Real Time PCR System (A, C, E, G) and SmartCycler II (B, D, F, H) instruments. A, C. Images show positive controls and samples with Ct lower than 38, respectively. B, D. Images represent melt curve analysis with Tm of 84°C for positive controls and samples, respectively. E, G. Images show no amplification for the negative controls and samples, respectively. F, H. Images show no amplification using melt curve analysis for negative controls and samples, respectively.

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