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LYMPHOGRANULOMA VENEREUM: A NEW FACE FOR AN OLD DISEASE.

Our experience in the Bologna area.

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INTRODUCTION.

Lymphogranuloma venereum (LGV) is a sexual transmitted infection due to Clamydia trachomatis biovar L, endemic in part of Africa, Asia, South America and the Caribbean, but rare in industrialized countries up to 10 years ago. In 2003, a cluster of cases of LGV among men who have sex with men (MSM) was reported in Rotterdam. Since then, several reports of LGV have been reported in the largest cities in Europe, the United States and Australia. They have usually occurred with an anorectal syndrome.

The purpose of this study is to summarize the expertise provided by the international literature about the new LGV outbreaks and to offer the first data collected on the presence of this disease in the Bologna area. In fact, we examine 5 cases of LGV proctitis diagnosed and treated at the Clinic of Sexually Transmitted Disease (STD) of the Dermatology Section at Sant'Orsola-Malpighi Hospital, Bologna. Particular attention will be paid to the laboratory method that allows identification and typing of the microorganism C. trachomatis serovar L1, L2, L3, leading to an etiologic diagnosis of certainty. The diagnosed cases of LGV will be described and compared with the international literature, trying to assess the risk factors, the most effective diagnostic and therapeutic procedure and the best approach to the patient.

CHLAMYDIA.

Classification.

Chlamydia are gram negative, not moving, round or oval bacteria; they are obligate intracellular parasites of eukaryotic cells. According to the classical taxonomy, they belong to the family *Chlamydiaceae* [1] that is comprised of the genus *Chlamydia* and four species, different from one another for phenotype, morphology and genetics: *C. trachomatis*, *C. psittaci*, *C.pecorum* and *C.pneumoniae* [2] (Figure 1).

Order	Chlamydiales
Family	Chlamydiaceae
Genus	Chlamydia
Species	*C. psittaci* *C. trachomatis* biovar LGV (L1-L3 serotypes) biovar Trachoma (ABC, D-K serotypes) biovar murine (MoPn agent) *C. pneumoniae* C. pecorum

Figure 1. Classical taxonomy of Chlamydia.

In 1999, Everett proposed a new classification based on a recent analysis of the 16S and 23S ribosomal DNA and on the identification of new intracellular microorganisms with a replicative cycle similar to Chlamydia. In this new classification, the order *Chlamydiales* includes four different families; the family *Chlamydiaceae* is divided into two genuses: *Chlamydia* and *Chlamydofila*; genus *Chlamydia* includes *C. trachomatis, C. muridarum* and *C. Suis* [1] (Figure 2).

Everett’s classification is still under debate [3].

In the *C. trachomatis* species, 19 serovars have been identified, divided into 2 biovars, trachoma and lymphogranuloma venereum (LGV). Serovars from A to K belong to biovar

trachoma, in which serovars A, B and C induce endemic thracoma; serovars from D to K induce urogenital infections, neonatal pneumonia and conjunctivitis. Biovar LGV includes serovar L1, L2, L3 [4].

Order	Chlamydiales		
Family	Chlamydiaceae		Simkaniaceae, Parachlamydiacee
Genus	Chlamydia	Chlamydophila	
Species	*Chlamydia suis* *Chlamydia muridarum* *Chlamydia trachomatis* MoPn LGV Trachoma	*Chlamydophila pecorum* *Chlamydophila psittaci* *Chlamydophila pneumoniae* TWAR Koala Equino *Chlamydophila abortus* *Chlamydophila caviae* *Chlamydophila felis*	

Figure 2. Taxonomy of Chlamydia proposed by Everett.

Cellular structure.

C. trachomatis is characterized by two cellular stages which alternate during the development cycle [5]: the elementary body (size 200-300 nm) which is incapable of replication but able to survive outside the host cell, and the reticular body (that reaches the size of 1000 nm), capable of replication but lacking the ability to infect. The reticular body replicates inside the infected cell and then it undergoes a reorganization process that transforms it into elementary body, which protrudes from the infected cell as a result of a process of lysis (Figure 3). Transmission therefore occurs through the elementary body, whether it be a sexual transmission or from mother to foetus.

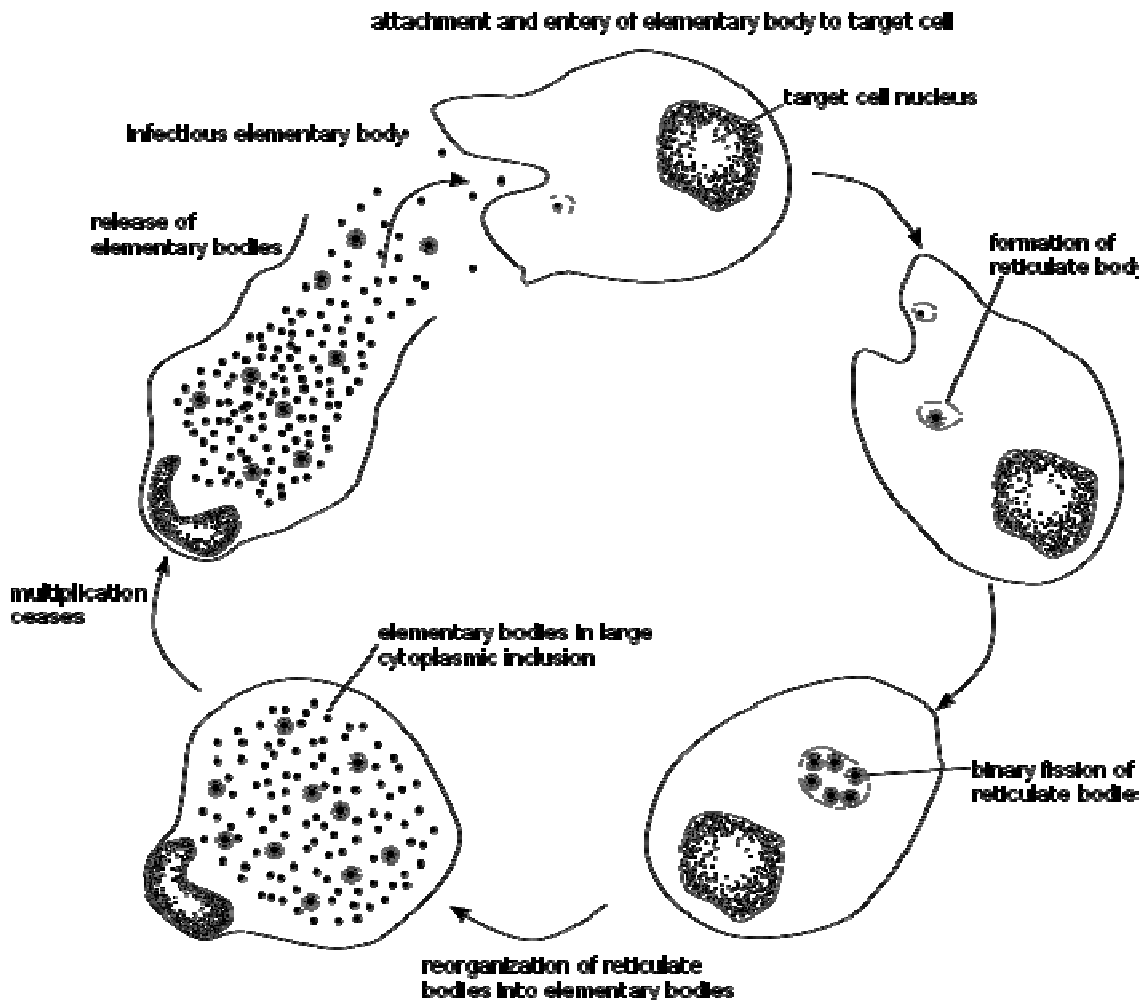

Figure 3. Infecting cycle of C. trachomatis.

Chlamydia presents a morphology similar to other gram – bacteria, but the cell wall does not contain any peptidoglycan (this is the cause of its resistance to ß-lactam antibiotics) [6,7].

Lipopolysaccharide (LPS) is localized on the outer membrane; the LPS is similar to that of enterobacteria, but it is equipped with lower cytotoxic activity, so as not to kill the host cell.

The outer membrane is made by the Outer Membrane Proteins (OMPs); about 60% of OMPs is represented by MOMP (Major Outer Membrane Protein), 38-40 kDa. MOMP presents genus-specific, specie-specific and type-specific antigens, targets of human antibodies; furthermore, it seems to play porinic function [8,9]. The other OMPs are two Cystein rich Proteins (CrP): the large CrP (60 kDa) and the small CrP (12 kDa). They are synthesized late during the replicative cycle and they are found in the membrane simultaneously to the appearance of the elementary bodies. This particular structure of the outer membrane seems responsible for preventing the fusion of fagosome with lysosome within the parasitized cell [10,11].

Infecting cycle.

The infection of an eukaryotic cell starts with the adhesion of an elementary body to the cytoplasmatic membrane. Elementary bodies possess an adhesion molecule similar to heparan-sulfate glycosaminoglycans (GAG) that binds a receptor expressed on cell susceptible to infection. Moreover, it seems that proteins of the outer membrane of the elementary bodies, equipped with negative electric charge at physiological pH, can consistently influence the infectivity of Chlamydiae [12]. The protein MOMP seems also to be involved in the adhesion process: treatment of the elementary bodies with trypsin, which determines partial digestion of MOMP, leads to the loss of the adhesive ability of Chlamydiae [13].

The entry of elementary bodies in the host cell takes place according to what has been called "phagocytosis determined by the parasite”: it seems a process of endocytosis conducted by the eukaryotic cell on the basis of a signal generated by the contact with the elementary body; energy consumption by Chlamydiae is not required. The *Tarp* protein seems to play an essential role in this process: it is a species-specific protein, translocated when the

elementary body contact the host cell; *Tarp* promotes the mobilization of actin and the modification of the chlamydial cytoskeleton [14]. Afterwards, the elementary bodies stay within the cytoplasmatic inclusions where they undergo a reorganization process over a period of 4-5 hours; their volume increase and, after 14-18 hours, they become reticular bodies, characterized by a reticular homogeneous structure observable under an electron microscope. The inclusions are unique vacuoles, different from the classical endocytic vacuoles; they do not undergo acidification or fusion with lysosomes, thanks to the ability of Chlamydiae of preventing the fusion of the phagosome in which they are contained with lysosomes. Probably, at this point, some relevant mechanisms favoring Chlamydiae survival are activated, as the modification of the endosomyc membranes, in order to allow the passage of nutrients [15,16]. Only C. trachomatis accumulates a significant amount of glycogen granules inside the inclusion; these granules are visible under the electronic microscope after staining with Lugol's liquid [17]. Between 18 and 24 hours after infection, some reticular bodies undergo a process of reorganization with a progressive reduction of their size, which leads again to the formation of elementary bodies. Then, the vacuolar membrane and the cytoplasm are digested by the cellular lysosomal enzime and Chlamydiae are released outside through cytolysis, starting a new infecting cycle.
About receptors on eukariotic cells, adhesiveness studies on HeLa and McCoy cells have shown that any serotype of C. trachomatis may be inhibited by another; this implies, at least for C. trachomatis, that the receptor present on eukaryotic cells is unique [18].

Metabolism, genetic and antigens.

Chlamydiae are able to synthesize autonomously several organic substances of low molecular weight and can provide for macromolecular synthesis by themselves, if the appropriate precursors are available. On the other hand, Chlamydiae are not able to produce energy (ATP, adenosine tri-phosphate) and this makes them obligate intracellular parasites [19].
The first genetic map of the genome of C. trachomatis was elaborated in the early 1990s. It permitted to estimate the genome of Chlamydiae in about 1.0-1.2 Mb, one of the smallest prokaryotic genomes which are able to encode approximately 600 proteins [20]. The homology of the chromosomal DNA between the three species of Chlamydiae is low and a

significant genetic variability was also found among some serotypes belonging to the same species of C. trachomatis [21].
In addition to the genome, some extra-chromosomal elements were identified in 1980 [22]. Some strains of *C. psittaci* and *C. pecorum*, almost all of the strains of *C. trachomatis* and only one equine strain (N16) of *C. pneumoniae*, contain a multicopy plasmid (5 to 10 copies) of 7.5 kbp, also called "cryptic plasmid", encoding eight ORFs (Oper Reading Frames) larger than 100 amino acids. The sequence of these plasmids is widely conserved within the different strains of C. trachomatis, although some cases have been described in which the same plasmid was missing without causing any functional consequence for the bacterium, suggesting that its presence is not essential for the survival of C. trachomatis. This plasmid is the target of molecular techniques for diagnosis of C. thracomatis. In fact, the presence within each single bacterial cell of 5-10 copies of plasmid amplifies the ability to detect a single bacterial cell, thereby increasing the sensitivity of these techniques [23].
Chlamydiae express genus-specific, species-specific and type-specific antigens. The main antigens are LPS, MOMP, responsible for most of the reactivity of sera in microimmunofluorescenze test, *large* CrP and a heat-shok protein of 60 kDa [24].

Pathogenetic mechanism.

Although Chlamydiae are obligate parasites with a clear cytolytic effect in cultured cells *in vitro*, evidence of a significant toxic effect *in vivo* is modest. During an acute infection, the infected mucous ephitelium presents an intense infiltration of polymorphonuclear cells, T-lymphocytes and B-lymphocytes, with consequent production of inflammatory cytokines. The immune response of the host seems to be involved in the genesis of mucosal damage [25].
The immune reaction is not able to quickly sterilize the focus of infection; prolonged inflammation may be able in time to suppress infection, but it may bring to a standing impairment of the tissues. In case of subsequent infections, the tissues impairment is often exacerbated by the chronic immune response and by the intense production of cytokines that stimulate fibrogenesis.
The host responses to Chlamydiae infection are different; they are responsible for resistance to re-infection and involved in the resolution of the disease. In the first phase of

the infection, the antibody response (IgG) is serotype-specific, preferentially recognizing the protein MOMP. In chronic or systemic infections supported by Chlamydiae, gender-specific antibodies against LPS and against *large* CrP appear. Although MOMP is the main antigen especially in infections with C. trachomatis and C. psittaci, sera of patients infected with C. pneumoniae may also recognize MOMP, possibly with a weaker reactivity [26,27].

C. trachomatis infections.

C.thracomatis includes 15 serotypes (or serovars), recognizable through antigenic differences of MOMP [28]:

- serotypes A, B, Ba, C cause endemic thracoma
- serotypes D, E, F, G, H, I, J, K are involved in genito-urinary infection in adults and conjunctivitis and pneumonia in newborns
- serotypes L1, L2, L3 cause lymphogranuloma venereum.

In addiction to these accepted serovars, some genovariants are described, as Da, Ja, L2a, L2b, L2c [29].

Chlamydiae non-L infections will not be topic of discussion in this paper.

C. trachomatis diagnosis.

Diagnosis of C. trachomatis infection is based on laboratory techniques. Possible samples are urethral and cervical swabs, first void urine, semen, rectal swabs, specimen collected from upper genital tract (Fallopian tubes, endometrium), buboes aspirate, nasopharyngeal and conjunctival specimen.

The following diagnostic techniques are available [30].

1. *Direct cytological examination*. This technique is useful in diagnosing acute conjunctivitis in newborns. It consists in a smear air dried and fixed with methanol, then colored with Giemsa; the diagnostic clue are intracellular basophilic and pinkish-blue inclusions.
2. *Isolation in cell culture*. It is the only procedure that confirms the presence of viable organisms. Clinical specimens, diluted in a suitable transport medium, are inoculated in pretreated cells (McCoy, Hep-2 and HeLa cells are most commonly used) and

centrifuged; after incubation for 48 to 72 hours, a cell monolayer is stained for intracytoplasmic inclusions with iodine staining or Giemsa or tested with fluorescent monoclonal antibodies binding MOMP's epitopes. Immunofluorescence is the preferred method and it shows a roundish intracytoplasmic inclusion. Culture is 80-85% sensitive and 100% specific, but time-consuming and it requires a laboratory equipped for cell culture.

3. *Antigen detection with immunoassays.* A number of commercial EIAs (Enzime Immuno Assays) are available for detection of chlamydial antigens. These tests use monoclonal or polyclonal antibodies to detect LPS; they are fast and simple to perform, but sensitivity for genital infection is 60-80% compared with the culture. False-positive results are possible because LPS is expressed by other bacteria.
4. *Serology.* Serology is not recommended for the diagnosis of chlamydial infection, with the exception of infection in newborns, in women with tubal infertility and occasionally in LGV when other tests are not available. In fact, all these more invasive syndromes are characterized by high levels of antibodies. Microimmunofluorescence (MIF) is the current method of choice. MIF is performed on a glass with suspension of elementary bodies of C. trachomatis, C. pneumonia and C. psittaci. Patients sera are tested against various antigens simultaneously and antibodies towards different chlamydial species are detected.
5. *Nucleic Acid Hybridization (NAH).* These tests has been widely used in the nineties and are based on the identification of specific DNA sequences with radioactive probes. Sensibility and specificity are similar to EIA.
6. *Nuclei Acid Amplification (NAA).* These tests seek for bacterial DNA (usually the target sequence of DNA is the "cryptic plasmid", that is substantially present in most common serotypes of C. trachomatis) by Polymerase chain reaction (PCR) or Ligase Chain Reaction (LCR), with the possibility of detecting the presence of a single elementary body. Nowadays NAA is considered the gold standard test for diagnosis of genital infection because its sensitivity has surpassed that of the culture. The disadvantages are the need of a laboratory equipped for molecular biology, the possibility of false-positive results and the high cost.

LYMPHOGRANULOMA VENEREUM

Lymphogranuloma venereum (LGV) is a venereal disease caused by C. trachomatis biovar L. It was described for the first time in 1833 by Wallace [31] and during the Nineteenth century it was considered a climatic disease, also named "tropical bubo" because it was more common among Africans; only in 1912 Rost identified its infective nature [32].
LGV is endemic in certain areas of Africa, Southeast Asia, India, the Caribbean, and South America. It is rare in industrialized Countries, but in the last 10 years it has been increasingly recognized in North America, Europe, and the United Kingdom as causing outbreaks of proctitis among men who have sex with men (MSM).

Etiology.

LGV is a systemic sexually transmitted disease caused by C. trachomatis serovar L1, L2, L3. L2 includes some variants, classified according to differences in amino acids of the gene *omp1*: L2a, L2b and the recently discovered L2c-g [33,34]. The recent spread of the disease in Western Countries is mainly driven by serotype L2b, identified in the first cluster of infection in Rotterdam in 2003 [35]. It seems that this Chlamydiae strain has been circulating in the Netherlands and presumably in Europe since 2000; moreover, it has been isolated retrospectively in rectal samples obtained from MSM in San Francisco during the period 1979-1985 [36]. This could suggest that L2b strain has been present for more than 25 years and the current LGV outbreak in industrialized Countries has most likely been a slowly evolving epidemic with an organism that has gone unnoticed in the community for many years and that is now being detected by new technologies.
A summary of LGV reports since the cases of San Francisco till the recent European outbreak is represented in Figure 4 [37].

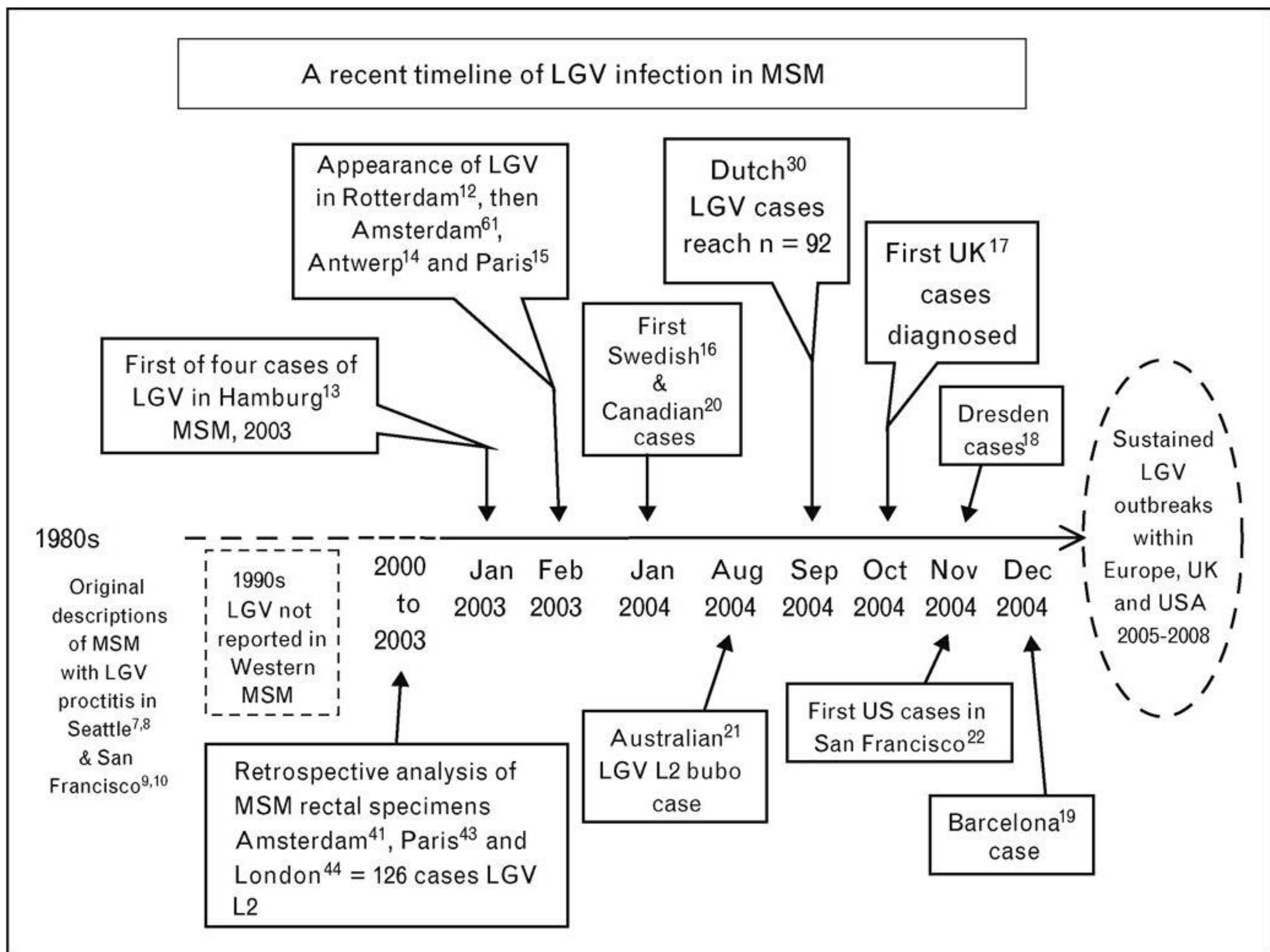

Figure 4. Published case reports of lymphogranuloma venereum in men who have sex with men occurring worldwide between 1981 and December 2004; cases from 2000 to 2003 were determined retrospectively.

Epidemiology.

The exact incidence of LGV is difficult to estimate; in fact, LGV is not subjected to mandatory reporting in most of the countries. Epidemiological surveillance is active only in some countries and it often considers data relating to high-risk population attending clinics for sexually transmitted diseases. Moreover diagnostic tests are not available in every laboratory and there are no screening programs.

The European Center for Disease Control reported about 1400 cases of LGV from 1990, but the cases were reported only from 16 countries where techniques for genotyping of C. trachomatis are available. The most affected age group is the 35-44 years group , followed by the 25-34 years group [38].

Several prevalence studies in special population are available.

Annan *et al.* found 36 rectal LGV infections in 3076 MSM in London during 2009 (prevalence 1,2%) [39]; in the same year, Ward *et al.* identified 61 cases in 6778 MSM patients attending 4 different clinics in the UK (prevalence 0,9%) [40].
A Dutch study on 1445 MSM patients found a prevalence of 3,1% [41].
In Australia, Templeton has obtained a prevalence of LGV infection of 0,3% in 2082 symptomatic or asymptomatic MSM patients [42].
A Finnish study found 9 rectal positive swabs for LGV on 1316 MSM (prevalence 0,7%) [43].
An American study has identified a prevalence of 0,87% in a group of 1671 Afro-Americans MSM participating in the *HIV Prevention Trials Network* [44].

Clinical presentation.

The classical clinical course of LGV consists of three stages [45]. During the first stage that usually occurs 3-30 days after inoculation, a painless papule erodes to form a small ulcer that heals rapidly with no scarring. The most common sites of infection are the coronal sulcus, prepuce, glans and scrotum in men, the posterior vaginal wall, posterior cervix or vulva for women; infection site may be also in rectal or pharyngeal mucosa, according to the kind of sexual contact. This transient ulceration, that often goes unnoticed by the patient, may be associated with urethritis or cervicitis.
The secondary stage begins 2-6 weeks after the primary lesion; it consists of painful inguinal lymphoadenopathy, often ipsilateral, if the inoculation site was on the skin of penis, vulva or perineum. Inflamed lymph nodes may coalesce to form buboes, that open on the skin in about one third of cases. Inguinal lymphoadenopathy is not appreciable in case of cervical or rectal infection; in these cases pelvic nodes are involved, causing unspecific abdominal pain or proctitis. Constitutional symptoms associated with the second stage include fever, chills, myalgias, and malaise; systemic spread of infection may occur with arthritis, ocular inflammatory disease, cardiac and pulmonary involvement, aseptic meningitis, hepatitis.
If infection is untreated, some patients may develop the third stage of disease: it is a chronic granulomatous inflammation that cause lymphatic obstruction, fistula and stenosis development, with severe functional impairment. Possible rectal complications are

perirectal fistulas, abscesses and rectal stenosis. Men with urethral infection may develop urethral stenosis and elephantiasis of the groin. Enlargement, thickening and fibrosis of the labia may occur in women, a condition termed esthiomene.
Despite a rectal involvement may rarely be present during the secondary stage even if the inoculation site was elsewhere, LGV proctitis is the main clinical presentation in the recent diffusion of LGV in men who have sex with men (MSM). It is characterized by anal pain, mucopurulent or hematic secretions, constipation or rectal tenesmus, usually occuring a few days after unprotected anal intercourse; inguinal lymphoadenitis is rare, since the most common site of infection is the rectum. A discrete ulcerative lesion may exist transiently in the rectal or perianal mucosa but no clear distinction is likely between 'primary' and 'secondary' proctitis, if anorectal transmission has occurred. LGV proctitis in MSM should be seen as a primary manifestation of infection following direct transmission to the rectal mucosa [45]. LGV proctitis may associate with constitutional symptoms, like reactive multi-joint arthritis [46].
During the last years, some authors have identified some cases of oro-pharingeal LGV [47,48,49], but identification of C. trachomatis serovar has not been obtained.
Although most of the recent cases of LGV present with proctitis, a small but significant number of inguinal-genital cases of LGV in MSM have been observed [50,51,52].
Despite the preponderance of rectal syndromes, the recent spread of the infection is not confined to LGV proctitis, but the 'classic' LGV is still present [53], being able to involve women [54] and heterosexual couples [55].
Finally, some cases of rectal or urethral LGV in asymptomatic patients have been identified [56,57].

Mode of transmission among MSM.

In the recent outbreak of LGV, the reported cases of LVG proctitis are many more than those of inguinal-genital syndrome; this fact suggests that transmission can occur from rectum to rectum, through fisting, sexual toys or enema [58]. Moreover, diagnostic techniques for C. trachomatis based on PCR on urine or on urethral swabs result negative in some patients with inguinal syndrome; in these cases C. trachomatis is more easily found in ulcer swabs or bubo aspirate; this could induce underestimation of classical LGV[52].

Risk behaviors for LGV proctitis are having unprotected receptive anal intercourses, using enemas and anal drugs, having sex in darkrooms, at sex parties and with HIV-positive partners [59]. It seems that rectal douching before intercourse for hygienic reasons could induce loss of integrity of the rectal mucosa, facilitating the transmission of infection, as already shown for hepatitis B [60] and hepatitis C [61].
Asymptomatic infections that might serve as reservoirs are rare. In a study conducted on 6778 rectal and 4825 urethral samples from MSM attending four genitourinary medicine clinics in the United Kingdom, only 4 out of the 63 positive samples have been from asymptomatic patients [58]. In a French study which has been performed recently on 2012 anorectal samples positive for C. trachomatis, 371 cases of LGV proctitis have been identified, 98% of which were symptomatic [62].
In addition, several studies trying to identify a significant reservoir of asymptomatic infection, urethral or rectal, in the general population, have failed [63,64], confirming that the infection appears to be restricted to the MSM population.
In the last twenty years of the Twentieth Century, LGV was rare or misdiagnosed in MSM; some authors suggests LGV was present but not diagnosed because genotyping of C. trachomatis was not currently performed[65] .
Another theory suggests that the spread of infection can be explained in the context of the immune reconstitution inflammatory syndrome (IRIS), characterized by paradoxical infections and inflammatory processes in immunocompromised HIV-infected patients, early after the beginning of the antiretroviral therapy. According to this hypothesis, the HIV-positive patients with asymptomatic LGV proctitis, would become symptomatic when their immune system responds to the antiretroviral therapy [66]. However, the same authors analyzed the association between having symptomatic LGV and the beginning of the highly active antiretroviral therapy (HAART) but no association was found and this hypothesis was subsequently disproved [59].
The changes in antibiotics requirements occurred in the last 20 years could be also considered. In fact, until the Nineties, most of the physicians used to treat urethral and rectal infections by Chlamydiae and nongonococcal urethritis with doxycycline for 7-10 days. Doxycycline was also used to treat unspecific proctitis and it was also given as part of many antigonococcal regimens to cover possible concomitant chlamydial infection.

Subsequently, over the past ten years, doxycycline has been gradually replaced with azithromycin in the treatment of chlamydial infection and nongonococcal urethritis. Azithromycin 1 gram in single administration has lacked to prove its efficacy in the treatment of LGV and rectal non-L chlamydial infection. If a short-course doxycycline therapy was effective at suppressing the spread of LGV infection for decades is still to be confirmed [44].

However, atypical presentation, lack of knowledge of the disease by physicians and patients and lack of diagnostic routine tests for chlamydial genotyping have lead to delays in diagnosis and treatment, impacting on the spread of infection in Western countries [67].

The current epidemic outbreak of LGV represents a significant public health problem, since LGV can facilitate both the transmission of HIV infection and other sexually transmitted disease [68].

Coinfection.

<u>HIV</u>

A strong association between LGV and HIV positivity is described by the literature: percentage of co-infection ranges from 67% to 100% across the reports [68]. In practice, two thirds of MSM with LGV are HIV-positive. The association between LGV and HIV may be linked to biological interactions between the two pathogens, but the microbiological studies that have been conducted up to now have not identified alterations in chlamydial infective cycle induced by HIV co-infection [69]. HIV-infection has been reported to cause abnormalities in the structure and function of the intestinal epithelial barrier function as well as abnormal lymphocyte trafficking that may facilitate LGV acquisition [70]. On the other side, LGV, as other ulcerative STDs, may increase both the acquisition and transmission of HIV infection [71].

The strongest confounder about the association LGV-HIV is high-risk sexual behavior. In fact, there have been behavioral changes in MSM communities as a response to HIV epidemic, and serosorting may make HIV-positivity an intermediating factor: individuals participate in certain type of practices partly because of their HIV-infection. Seroadaptive behavior can reduce the number of new HIV infections but it also facilitates the spread of other STIs inside a specific network [68].

HIV infection does not change clinical manifestation of LGV and does not alter the response to antibiotic treatment [59].

HCV

During the last decade, outbreaks of acute hepatitis C virus (HCV) infection have been reported among HIV-infected MSM in several European countries. In most of the cases diagnosis of HCV is strictly preceded by diagnosis of an ulcerative STD, as syphilis or LGV [72].

LGV and HCV probably share several high-risk behavior, as rough sexual intercourse, traumatic practices and use of drugs.

In the literature, co-infection between LGV and HCV reaches a prevalence of 19% in some studies [73].

Diagnosis.

Currently, a diagnosis of certainty of LGV infection may be currently achieved only by detecting the biovar-specific C. trachomatis DNA with molecular biologic tests. These techniques may be applied on biological samples taken from the primitive anogenital ulceration, from the bubo aspirates, if present, or on anorectal swabs, preferably collected during proctoscopy, but also a blind anorectal swab can suffice.

Biological samples first undergo a commercial C. trachomatis NAAT test identification; although commercially available tests are not approved for extragenital sites, a large body of literature supports the use of these tests for the detection of rectal chlamydial infections [66,74,75,76]. If the sample is positive, the biovar-specific DNA is identified with a Home-made PCR; at the beginning , a real-time PCR-based test that specifically detects all C. trachomatis LGV biovar strains was available [77] and more recently, a real-time quadriplex PCR-based assay which incorporates both LGV-specific and non-LGV-specific target sequences, a C. trachomatis plasmid target, and the human RNase P gene as an internal control[78].

If biologic molecular tests are not available, a presumed diagnosis may be based on clinical symptoms supported by chlamydial-specific serology, especially a high titre of IgA anti-

MOMP antibodies. However, a low IgA titre does not exclude the diagnosis and high IgA level in asymptomatic patients does not confirm LGV infection [35,66].

Histopathology of mucosal manifestations is not specific; it usually shows a chronic, mainly lymphocytic, inflammatory infiltrate and hyperplasia of lymphatic follicles [79].

Microscopic examination of rectal smear test may raise the suspicion of LGV proctitis if it shows an increase number of polymorphonuclear leukocytes (>10-20 cells/high power field); this finding does not exclude a gonococcal proctites [45].

A proposal to define certain, probable or possible LGV infection [80] is summarized in the table below (Figure 5).

Criteria	**Classification**		
	Confirmed	*Probable*	*Possible*
LGV pathology or contact with LGV patient	Yes	Yes	Yes
C. trachomatis serology, invasive titres	Positive/unknown	Positive	Positive
C. trachomatis PCR (urine/rectum)	Positive	Positive	Unknown
C. trachomatis PCR genotype serovar L1–L3	Positive	Unknown	Unknown

Figure 5. Lymphogranuloma venereum (LGV) case definition proposed and used in the Netherlands since January 2004.

Differential diagnosis.

The main differential diagnosis are other ulcerative STDs, as herpes simplex, non-LGV Chlamydia, gonorrhoea and syphilis, chronic bowel inflammatory diseases and rectal carcinoma.

Anorectal herpes simplex usually presents with mucosal ulcerations and pain and it should be excluded by Tzank smear examination or by specific PCR [81].

Non-LGV Chlamydia proctitis is usually slightly symptomatic; diagnosis is based on biovar-specific DNA identification [81].

Gonococcal proctitis provokes mucopurulent discharge, pruritus or pain. A gram stain of discharge showing gram-negative diplococcus may point out the diagnosis; culture is the

preferred diagnostic techniques, since it also provides information about antibiotics susceptibility; NAAT is also a sensible and specific test [81].
Primary syphilis can occur with anal ulcerations with discharge and pain; a non-treponemal serologic test (VDRL or RPR) and a specific treponemal antigen test (FTA-ABS) must always be performed in patients with suspected infective proctitis [81].
LGV proctitis may be in the form of inflammatory bowel disease in MSM. In fact, clinical endoscopic and histological findings in LGV can be indistinguishable from those of IBD, as Crohn's disease or ulcerative recto-colitis. LGV proctitis may be misdiagnosed and treated with anti-inflammatory therapy with the risk of late complications. A rectal swabs for C. trachomatis NAAT is recommended in case of proctitis, especially in MSM. If C. trachomatis NAAT is not available, LGV proctitis should be considered in case of IBD treatment failure, in HIV positive or MSM patients, if Chlamydial serology is positive and in case of healing after treatment with doxycycline [82].
Rectal localization of LGV can be confused with colon-rectal cancer; in case of a rectal neoformation with histological examination negative for malignancy, C. trachomatis infection has to be ruled out [79].

Treatment.

Despite the lack of controlled randomized studies, current guidelines recommend oral doxycycline 100 mg twice a day for 21 days to treat LGV; most of the reported cases by the literature obtained complete response with this treatment, also in HIV co-infection.
Second line treatment is oral erythromycin 500 mg four times a day for 21 days.
Azithromycin 1 gram in single or multiple doses has been proposed, but there is no evidence to recommend this therapy.
Fluctuant buboes should be aspirated through healthy adjacent skin, while surgical incision is not recommended due to potential complication such as chronic sinus formation.
A complete screening for STDs should be proposed in each case of LGV.
Sexual contacts of the last 3 months should be screened for LGV and empiric antibiotic treatment should be started until LGV has been excluded.
A test of cure for LGV is not necessary if treatment with doxycycline is completed [83].

MOLECULAR BIOLOGY.

Molecular biology techniques available to point out the nucleic acids of microorganism in biological samples are the nucleic acid hybridization (NAH), that use probes to identify a specific target, and the nucleic acid amplification (NAA). NAA is the most used because of its greater sensibility.

Both NAH and NAA begin with the same procedure, the extraction of nucleic acids.

Extraction of nucleic acids.

Extraction of nucleic acids (DNA or RNA) from biologic samples consists in purifying and concentrating the nucleic acids, removing potential inhibitors of amplification reaction. The kind of biological sample influences difficulty and price of the extraction process: blood, plasma, cerebrospinal fluid require an easier process than feces, sputum, aspirates or bones.

Furthermore, the kind of microorganism also influences extraction: fungi and mycobacteria are more complicated than viruses.

Extraction of nucleic acids is mainly based on separation methods with organic solvents, methods for affinity or ion exchange. Automated systems for the extraction of nucleic acids from clinical samples (serum, plasma, urogenital swabs, urine, cerebrospinal fluid, etc. ..) with standardized rapid and simple methods, have been commercially available for a decade. Moreover, these automated systems are equipped with an internal control, that is subjected to the same treatments of the biological sample, thereby demonstrating their validity. The automated systems exploit the principle of affinity of the nucleic acids to the silica, which is located on the surface of magnetic beads (Figure 6), or the bond to DNA/RNA capture probes: in the latter case specific probes for nucleic acids are bonded to the magnetic beads [84].

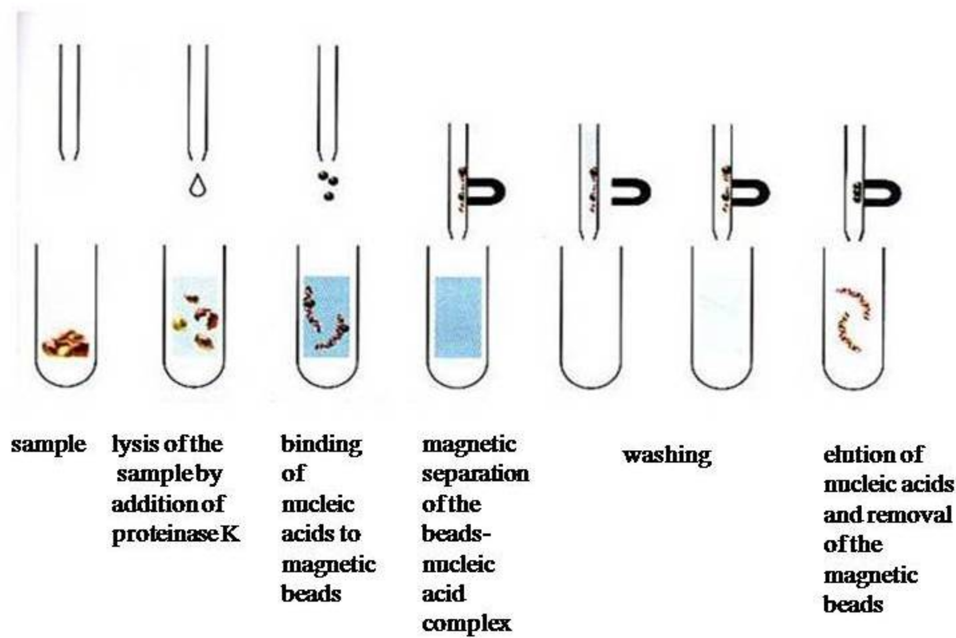

Figure 6. Automated extraction of nucleic acids exploiting affinity to silica.

Nucleic acids amplification.

The nucleic acid amplification techniques are PCR (Polymerase Chain Reaction), TMA (Transcription-Mediated Amplification), NASBA (Nucleic Acid Sequence-Based Amplification) and LCR (Ligase Chain Reaction). PCR is the most used both for research and diagnostic purposes.

PCR, invented by Kary Mullis in 1985, is a quick, sensitive and flexible method to amplify in vitro predetermined sequences of DNA or RNA, generating millions of copies, from an initial complex source of nucleic acids.

PCR requires two short sequences (primers) complementary to the sequences flanking the stretch of DNA segment to be amplified. The enzyme DNA polymerase is able to synthesize a DNA strand starting from the same pair of primers (short DNA sequences that hybridize at two different point of the DNA sequences), using the opposite strand of DNA as template. In order to use PCR for diagnostic purposes it is necessary to know a genomic sequence of the microorganism to be used as a target; in this way two specific primers, hybridizing respectively in two different points of the sequence of the target DNA, can be synthesized.

In a first step it is necessary to ensure the separation of the two DNA strands (denaturation step), then the creation of the link between the primers and their complementary regions of the filaments of denatured DNA (annealing step). Finally, the reaction of the polymerase (extension phase of the filament from primers 5') may start. Polymerases usually belong to thermophilic organism and are not inactivated by high temperatures, such as the Taq polymerase from the thermophilic bacterium *Thermus aquaticus*. This allows to realize more PCR cycles in sequence; in each cycle the DNA synthesized in the previous steps is also duplicated, obtaining a chain reaction that allows an extremely rapid multiplication of the genetic material of interest.

During the denaturation step, the solution of DNA to be replicated, deoxyribonucleotide triphosphates (dNTPs), magnesium ions, primers and DNA polymerase, is brought to a high temperature (over 90° C); the double helix of DNA is cleaved and the two strands which constitute it are released.

Subsequently the temperature is lowered to 40-55° C, in order to allow the binding of the primers to their complementary regions of the filaments of denatured DNA (annealing step).

Finally, the temperature is raised up to 65-72 ° C in order to maximize the action of DNA polymerase that determines an elongation of the primers linked, using as template the single-stranded DNA (extension phase) (Figure 7).

The described cycle is repeated typically for about 30-40 times. Generally the process does not exceed 50 cycles because at a certain point the amount of DNA obtained reaches a plateau.

The greatest problem of PCR derives from its high sensitivity and efficiency; in fact, PCR is very sensitive to the presence of contaminant genetic material that can be found on instruments, on operators, or in the external environment. The major source of contamination is the opening of tubes containing amplified material, which can spread in the air as aerosols and contaminate subsequent PCR [84].

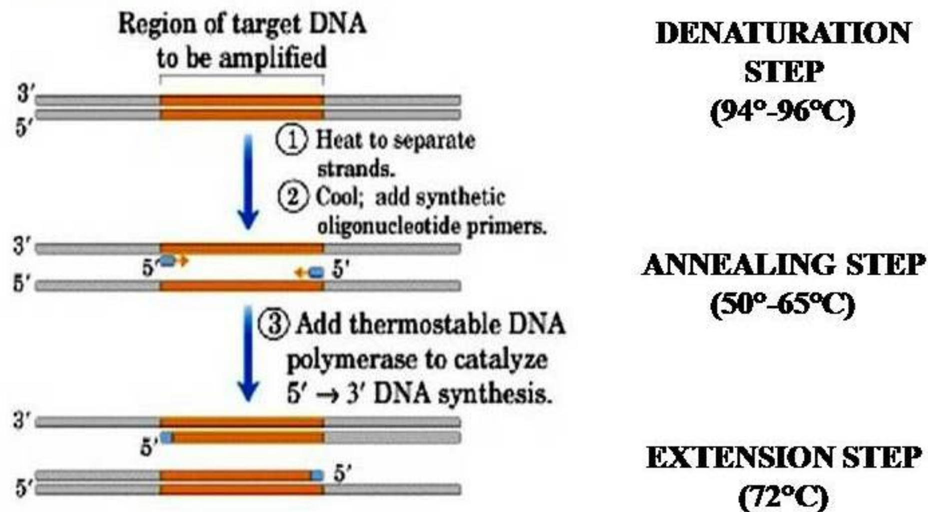

Figure 7. Thermal cycles of PCR.

Post amplification detection.

Different methods for detection after amplification are available; the choice of the right method is based on the characteristic of the amplified product and if there is the need to sequence the product. Another factor to consider is the cost of the equipment required for detection. In daily use, diagnostic detection of PCR products on agarose gel or by blotting methods are giving way to new automated methods (Real-Time PCR).

<u>Electrophresis on agarose gel.</u>

The agarose gel electrophoresis is a simple and fast method that allows to separate, and then identify, DNA fragments according to their molecular weight. The agarose gel is subjected to an electric field that makes the DNA migrate from the negative pole to the positive one, as a function of the electrical charges conferred by phosphate groups. The migration speed depends on the size of the fragments, the percentage of agarose in the gel and by the applied voltage; smaller linear fragments migrate faster than larger ones. Several types of dyes are used to highlight the PCR products on gel; the most widely used is the ethidium bromide, that is a planar molecule which fits between the bases of the double stranded DNA and emits fluorescent light when irradiated with ultraviolet light (300 nm). Unfortunately the

ethidium bromide is considered as one of the most dangerous mutagenic agents present in the laboratory; therefore in recent years new safer methods for staining of the agarose gel has been searched [84].

<u>Capture of the amplification product.</u>

In this detection method, probes specific for the amplification product to detect are bonded to 96-well microplates or magnetic beads. During the amplification step of PCR, primers are linked in 5' to a molecule of biotin; the biotinylated DNA binds to the probe and, after several washes to remove the non-specific products, a substrate is added and the resulting colorimetric reaction is read at spectrophotometer [84].

Real-Time PCR.

Real-Time PCR is a technological innovation, characterized by real-time detection of the amplified product and by the ability to multiple searches. In fact, thanks to the new Real-Time techniques, it has been possible to develop “multiplex tests”, which allow the simultaneous amplification of multiple DNA sequences due to the presence, in the reaction mixture, of more primer pairs, each specific against a particular genomic region. In this way it is possible, at the same time, to identify different pathogens in the same sample or amplify different genomic regions of the same organism.

In traditional PCR, in each cycle the three phases of DNA denaturation, annealing and extension are repeated. This process occurs inside the tubes where the target DNA and the mixture of primers, Taq and dNTPs, are located; the tubes are loaded at the beginning of the operations in the thermocycler which is able to vary continuously the temperature depending on the different phase of each cycle. At the end of the amplification, detection of PCR products is required, with the electrophoretic run on agarose gel or with methods of capture on plate.

In Real-Time PCR, a single system serves both as thermocycler and as detector of the amplified product. The main advantage of Real-Time PCR is the reduction of contamination by carry-over, since amplification and detection are performed inside sealed plates or tubes and leakage of the amplified DNA is avoided [84].

The most Real-Time PCR in commerce are based on the transfer of fluorescent energy by resonance (Fluorescent Resonance Energy Transfert, FRET). FRET indicates transfer of energy between two neighboring molecules, which act as markers; one acts as a donor and the other as an energy acceptor. The acceptor may be a fluorescent molecule or a non-fluorescent molecule able to switch off the signal of the donor (in this case is called quencher). The markers are attached to small DNA probes specific for the sequence of interest. The efficiency of energy transfer between the two markers depends on the distance that separates them and the degree of overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor (quencher). DNA probes for Real-Time PCR with the use of a quencher are among the most widely used, because the quencher molecules have broad spectra of absorption with low basal levels of fluorescence: these characteristics allow the use of a single quencher for most donors, each with its own specific wavelength emission.

The main Real-Time PCR system available today involves the use of TaqMan probes. This method exploits the ability as exonuclease 5 '→ 3' of Taq enzyme to generate a specific product that can be revealed simultaneously to the amplification.

TaqMan probes hybridize within the target sequence; the donor (for example a molecule of carboxyfluorescein, FAM) is linked in 5’ of the TaqMan probe, while the quencher (a non fluorescent molecule or a molecule with different fluorescence) is linked in 3’. TaqMan probes do not participate in the amplification because they are phosphorilated in 3’ in order to prevent their extension by Taq. The emission of light by the donor is blocked by its proximity to the quencher. During the extension phase from one of the primers, Taq cuts nucleotides in 5' of the TaqMan probe thanks to its exonuclease activity 5' → 3 '; thereby FAM is released by the quencher. Now FAM emits fluorescence, while the quencher is not changed by the cut. Since the exonuclease activity of the Taq only occurs if the TaqMan probe is attached to the target, the increase of fluorescence is directly proportional to the quantity of PCR products formed: the system is able to quantize the number of amplified fragments of DNA that are formed in the PCR reaction [84] (Figure 8).

The evaluation of the amount of the target DNA is performed by determining the threshold cycle, in which the threshold value of fluorescence is reached, that is when specific amplification signals are separable from the background noise of the system. The number of

cycles necessary to reach the threshold cycle is inversely proportional to the number of targets present in the initial sample.

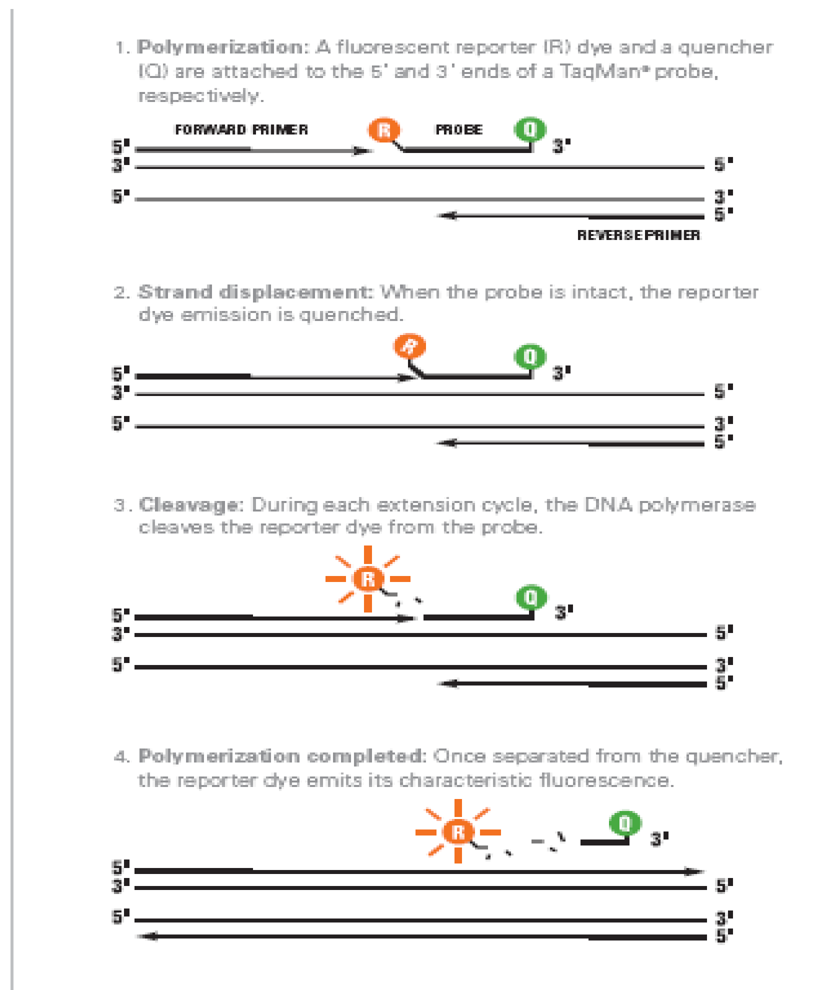

Figure 8. Operating diagram of Fluorescent Resonance Energy Transfert, FRET, with TaqMan probes.

C. trachomatis genotyping.

Since C. trachomatis was first isolated in 1957, extensive efforts have been made towards development of methods to characterize this important pathogen into different types. In the 1960s, Wang and Grayson developed the mouse toxicity prevention test (MTPT), inoculating egg yolk sacs with "trachoma-inclusion conjunctivite organism", then injecting extracts of these into mice. Subsequently, intravenous injection of the same or different

isolated of Chlamydia were repeated in the same mice and a protective mechanism in mice that have received the same isolated, or a partial cross-protection from other isolates, was reported. In this way, the authors demonstrated the presence of different kind of Chlamydia and classified 80 strains of Chlamydia into six immunological types [85].

Looking for a faster and simpler test, the same authors developed in the 1970s a microimmunofluorescence test, based on elementary bodies of known type grown in yolk sacs and polyvalent antiserum produced in mice [86]. When cellular culture became applicable, in the 1960s, researchers identified 13 different types of Chlamydia, according to the surface antigens A, Ba, B, C, D, E, F, G, H, I and L1-3 [86,87]; types Ia, J,Ja and K were identified later. These classification in 17 different serotypes is still in use.

Microimmunofluorescence was hampered by cross-reactivity between related types and the polyclonal antibodies were later replaced by more specific monoclonal antibodies. It was recognized that the main epitopes for antibodies were located on the MOMP (Major Outer Membrane Protein) and all the subsequent typing procedures were based on this protein or on the gene *omp1* encoding for it.

Then, new tests based on monoclonal antibodies were developed and antibody panels became commercially available; the first studies on tissue tropism of serovars were published.

All the above mentioned techniques require culture of C. trachomatis, which is expensive and time consuming. Moreover, serovars unrecognized by the antibody panels might evolve and change over time. Finally, sensitivity of culture is often less than for the NAAT assays [88].

PCR and Restriction Fragment Lenght Polymorphism (RFLP).

With the invention of PCR in 1983, the era of genotyping began. In the 1990s, PCR was often followed by RFLP, with DNA patterns resolved on gel. The patterns were found to be compatible with the results obtained with serotyping. In 1991, Rodrigez *et al* amplified 1200 bases of the *omp1* gene and the product was digest by restriction nuclease and resolved on polyacrylamide gels [89]. Thirteen of the 15 serovars could be differentiate by this technique; from there, several studies extended and improved batteries of restriction enzymes.

Omp1 is a single copy gene and to obtain adequate material for RFLP, PCR was improved using nested primers. A nested PCR consists in a second PCR carried out on the products of the first PCR, using primers complementary to regions of the same DNA fragment, but more internal compared to the regions of annealing of the first primer pair.
In the first report about RFLP, it was point out that serovar E is the most prevalent type in heterosexual population, followed by serovars D and F. On the contrary, in MSM, the prevailing serovars are G, D and J.
From 1993, it was shown that PCR, and subsequent typing, could be made directly from biologic samples, avoiding the need to use cell culture.
The strength of PCR and RFLP method is its ability to detect mixed infections; this ability has been overestimated in the past, in fact PCR tend to favor the most represented serovar and the others may remain undetected. The drawback of this method is that it does not allow for identification of single nucleotide changes, which are the most predominant variations in *omp*1.
PCR and RFLP are still often used, also for diagnosis of LGV [88].

<u>PCR and sequencing.</u>
With automation and the availability of fluorophore-marked nucleotides, it has become possible for routine laboratories with no culture facilities to perform sequencing. It has been shown that sequence determination of *omp1* provided a higher resolution than serotyping and RFLP [90,91,92]. The main drawbacks of sequencing are the difficulty in identifying mixed infections and the inadequate ability to discriminate between different genotypes, because peaks from different PCR products will be superposed in the chromatograms from sequencing reactions. These defects do not make it suitable as a reference method for genotyping in unselected populations. In a recent study, Simpson's index of diversity (D) for *omp1* has reported to be 0.69, an extremely lower value than the 0.95 required by the guidelines of the European Society of Clinical Microbiology and Infectious Disease (ESCMID) and the European Study Group on Epidemiological Markers (ESGEM) for a typing system [88].

Other typing methods.

Other methods have been developed for genotyping of C. trachomatis using *omp1* as target.

- For the three L-serovars, genotyping has been enabled by Real-Time PCR targeting the *omp1* gene and an L-serovar specific gap in the polymorphic protein H (*pmpH*). Differentiation between the three L-serovar is obtained by a second Real-Time PCR targeting the serovar-specific regions of the *omp1*. The main advantage of this method appears to be the ability to detect multiple infections, but on the other hand two consecutive PCR must be carried out for a complete genotyping [93].
- Pulsed field gel electrophoresis (PFGE) requires pure bacterial DNA taken from Chlamydia culture and purified from the host DNA. This method has been used to create a genetic map of the serovar L2 [94], but they have minor ability to discriminate against the different strains than serotyping and genotyping based on gene *omp1*.
- Random amplification of polymorphic DNA (RAPD), despite not reflecting the serotyping, can be used as a supplement to *omp1* typing [95].
- The technique of amplified fragment length polymorphism (AFLP) uses restriction enzymes to cut the genomic DNA, followed by amplification of restriction fragments then displayed on gel. This method requires the culture of the genomic DNA and purification of the elementary bodies; it is not easy to reproduce [91].
- The Microsphere Suspension Array allows the identification of more than 100 biomolecules, including proteins and nucleic acids. When accompanied by a multiplex PCR with a sensitivity comparable to the single-plex assay, with a high number of primers, the system is simple to use, inexpensive and allows to optimize time compared to the PCR followed by sequencing, allowing also to identify multiple infections [96].
- A low-resolution typing system has been developed thanks to the Reverse Line Blot assay, which allows a faster and less laborious genotyping compared to gene sequencing, but may miss subtle changes in *omp1*, for example point mutations, that are the most frequently found alterations in this gene [88].

None of the tested target genes provide sufficient discrimination of C. trachomatis strains, and there is need for better genotyping system. Two recent alternative detection system seemed to provide high resolution typing of C. trachomatis.

- A combination of PCR and sequencing of *omp1* plus three loci of VNTR (Variable Number Tandem Repeat, that are repeated stretches of the same nucleotide or motifs) is called MLVA (multiple loci variable number of tandem repeats analysis); this approach gets a Simpson's index of diversity of 0.94, very close to the recommended value of 0.95. This method is by far more discriminative than *omp1* alone (D=0.69) but, such as other PCR and sequencing assays, is unable to detect mixed infections. The analysis of VNTR targets will result in high resolution power especially for short-term, local epidemiology, since in these areas of repetition DNA polymerase is prone to error during replication. However, MLVA is rarely used as a diagnostic method for routine, playing an important role as a final method when other typing methods have failed [97].
- The multilocus sequence typing (MLST) is a genotyping method based on amplification and sequencing of several genetic regions. The previous studies and systems based on single genes as targets for typing have shown serious limitations due to the low variation of sequences, probably due to the genetic isolation of Chlamydia and the low degree of horizontal gene transfer during evolution. MLST systems have been developed to understand the genetic structure of the population and to evaluate the association between genotype and disease. A kind of MLST reported by Klint *et al*, includes 5 highly variable gene regions; 2 of them (penicillin-binding protein and histone H1–like protein [*hctB*]), are subjected to selection pressure, which facilitates the analysis of epidemiologic changes over limited periods[98]. Albeit with some limitations, this system can be used on clinical samples from NAAT diagnostic and possesses a discriminatory ability high enough to be used to study the contact-tracing.

  An alternative MLST system has also been based on 7 housekeeping genes and analyzes evolutional changes rather than acting as a tool for partner notification [88].
- Multilocus Typing DNA Microarrays, an alternative technique to MLST, has been proposed recently. It is a faster and cheaper system and it proves to have a detection

capability which is almost twofold higher than typing base on *omp1*; moreover it allows identification of mixed infections.

All the techniques described above and actually used require the amplification of a target by a PCR and therefore depend on it in terms of sensitivity and specificity.
Despite the important epidemiological knowledge which has been obtained through serotyping, genotyping based on PCR and sequencing of *omp1* is now replacing serotyping. The insufficient epidemiologic resolution achieved by characterization of *omp1*, makes it necessary to apply new genotyping methods, as MLVA or MLST. However, it is important to underline how the culture of C. trachomatis can still play an important role in the identification and characterization of new variants and how the methods of typing based only on *omp1* are still often used for studies of molecular epidemiology [88].
The main features of the different methods of typing are schematically summarized in Figure 9.

Finally, these are the principal hand points and reasons for typing [88]:

- to study the transmission pattern in sexual networks
- to be used as a tool in partner notification
- to enable association with clinical manifestations and pathogenicity
- to study association between specific serovars and tissue or organ affinity
- as an aid in cases of sexual abuse
- to help determine whether infections are persistent or new
- to monitor treatment outcomes
- to develop future vaccines
- for evolutionary surveillance of specific clones.

Method	Mechanism	Target	Reference	Culture	Comment
MTPT	Immunization	Unknown	Wang & Grayston (1963)	NA	Proof of existence of different types
Microimmunofluorescens	Immunization	MOMP	Wang et al. (1975)	NA	Cross-activity between types
Polyclonal antibodies	Serotyping	MOMP	Wang et al. (1973b)	Y	Cross-activity between types
Monoclonal antibodies	Serotyping	MOMP	Stephens et al. (1982) Wang et al. (1985)	Y	Cross-activity between types
RIA	Serotyping	MOMP	Van Der Pol & Jones (1992)	Y	Cross-activity between types
Dot-ELISA	Serotyping	MOMP	Barnes et al. (1987)	Y	Cross-activity between types
PCR-RFLP	Genotyping	Omp1	Rodriguez et al. (1991)	Y	13 of 15serovars identified
PCR-RFLP	Genotyping	Omp1	Lan et al. (1993)	Y	Cervical scrapes used
PFGE	Genotyping	Genomic DNA	Birkelund & Stephens (1992)	N	Purification of EBs needed
RAPD	Random DNA	Genomic DNA	Scieux et al. (1993)	NA	Not reflecting serovars
PCR	Genotyping	Omp1	Pecharatana et al. (1993)	Y	Fluorophore marked primers Only serotypes A, B, C detected
AFLP	Genotyping	Genomic DNA	Morre et al. (2000)	NA	Difficult to standardize
Reverse line blot	Genotyping	Omp1	Quint et al. (2007)	Y	Low technology detection
PCR+sequencing	Genotyping	Omp1	Lysen et al. (2004)	N	Serotype-level typing
Real-time PCR	Genotyping	Omp1 Pmp-H	Schaeffer & Henrich (2008)	Y	Only L-serovar
PCR+sequencing	Genotyping	MLST	Klint et al. (2007)	N	Subtyping serovars
PCR+sequencing	Genotyping	Omp1 VNTR	Pedersen et al. (2008)	N	Subtyping serovars
Suspension array	Genotyping	Omp1	Huang et al. (2008)	Y	Serotype-level typing

High resolution Melting analysis	Genotyping	Omp1	Li et al. (2010)	N	Serotype-level typing
Microarray analysis	Genotyping	Omp1	Christerson L et al. (2011) Ruettger A et al. (2011)	N	Serotype-level typing Identifies mixed infections

Figure 9. The main features of different methods for typing (MTPT Mouse toxicity prevention test ; RIA Radio immunoassay; ELISA Enzyme-linked immunoadsorbent assay; RFLP Restriction fragment length polymorphism; PFGE Pulsed-field gel electrophoresis; RAPD Random amplification of polymorphic DNA; AFLP Amplified fragment length polymorphism; NA non applicable).

EXPERIMENTAL SECTION: OUR EXPERIENCE IN THE BOLOGNA AREA.

Since 2009, routine diagnosis of Chlamydia trachomatis and Neisseria gonorrhoeae genitourinary infection in patients attending the Clinic of Sexually Transmitted Disease (STD), Dermatology Section , Sant' Orsola-Malpighi Hospital, Bologna has been carried out by a commercial Real-Time PCR (VERSANT® CT/GC 1.0 Assay). The Section of Clinical Microbiology of our hospital has developed a home-made PCR test recently in order to typify C. trachomatis serovars non-L and serovars L.
So far, the diagnosis of lymphogranuloma venereum (LGV) for patients attending our Clinic has been based on clinical symptoms and signs and it has been supported by chlamydial-specific serology; an etiologic diagnosis of certainty has not been possible. The assessment of response to the treatment was also mainly based on the resolution of clinical symptoms.
The development of the new diagnostic technique allows a diagnosis of certainty of LGV through identification and typing of C. trachomatis serovars L.
The purpose of our study is to use the new diagnostic test to identify accurately LGV infection in our patients, also in relation to the ongoing outbreak of LGV proctitis in MSM in industrialized countries. The diagnosed cases of LGV will be described and compared with the international literature.

Patients and methods.

From January 2012 to January 2013, patients attending our STDs Clinic complaining of rectal disorder or simply with a positive anamnesis for unprotected anal intercourse in the last 6 months were asked to carry out a clinical visit, a rectal swab and a sample of urine to detect C. trachomatis and N. gonorrhoeae infections using a Real-Time PCR (VERSANT® CT/GC 1.0 Assay). All positive samples for Chlamydia were typified with a home-made PCR to identify the specific serovar.
During the clinical visit, the external genitalia, perianal skin and anal mucosa were evaluated; in particular the presence of perianal ulcerations, anal discharge and inguinal

lymphadenopathy were assessed. If genital or perineal ulceration were present, HSV infection were evaluated with Tzank smear examination.

All patients underwent a complete blood screening for the main STDs (HIV, HBV, HCV, syphilis) and for chlamydial serology (anti-Chlamydia IgA ang IgG determined with an immunoenzimatic test).

Furthermore, personal details (sex, age, nationality), data about subjective urogenital and gastrointestinal symptoms, sexual behavior, habit of using condom, number of sexual partners in the last 6 months and previous STDs were collected for each patients.

Patients with LGV were treated and re-evaluated with clinical examination and laboratory tests (VERSANT® CT/GC 1.0 Assay on urine and anorectal swab, chlamydial serology) 3 months after the antibiotic treatment.

Patients took part in this study after having given their consent.

Diagnosis of C. trachomatis infection with Real-Time PCR.

VERSANT® CT/GC 1.0 Assay (Siemens Healthcare Diagnostics, USA), based on a procedure of kinetic PCR (kPCR), is a fully automated qualitative *in vitro* test for the detection of nucleic acids of C. trachomatis (CT) and N. gonorrhoeae (GC) in clinical samples of both symptomatic and asymptomatic patients [99].

The VERSANT kPCR system is composed of a module for the preparation phase and a module for amplification, managed by a dedicated software (Figure 10). The extraction phase is totally automated and works binding the nucleic acids to the surface of magnetic beads coated with silica. In the module for the extraction the automated dispensing of the mixture of primers, probes, dNTPs and enzyme Uracil DNA Glycosylase AmpErase ™ UNG (1 unit / mL) also takes place in order to diminish the environment contamination by the amplified products. Moreover, during the extraction phase, proteinase K inactivates the DNAse and lyses the bacterial cell allowing the release of DNA. The DNA released from bacterial particles, along with other nucleic acids and internal control specially added, binds to silica of the magnetic beads. A number of successive washes remove the unwanted components of the sample. The purified DNA is eluted and subsequently loaded into a 96-well plate for the execution of the kPCR.

A mixture of probes (Taqman), primers and dNTPs is added to each well. The nucleotide bases A, G, C and U, instead of the traditional base T, are contained in the mixture. The target sequences of C. trachomatis, as well as the internal control DNA, are amplified and detected simultaneously during the Real-Time PCR. Chlamydia TaqMan PCR primers and probes were designed to target the Gen Bank nucleic acid sequence of the 7.5 kb cryptic plasmid outside of 377 bp deletion, being so able to identify also the nvCT mutant [99].
In the preliminary phase, the enzyme Uracil DNA Glycosylase AmpErase ™ UNG is left to act for 10 minutes at 50°C, then it is inactivated for 15 minutes at 95°C. After this, the real PCR begins and it is repeated for 40 cycles.
Each cycle consists of two parts: during the first one, at 95°C for 15 seconds, denaturation of nucleic acids occurs; during the second one, at 62°C for 1 minute, annealing and elongation of the sequences to be amplified take place.
The probes include fluorescent tracers and quenchers as modifiers. The probes, labeled in this double way, specifically detect the presence of amplified originated from C. trachomatis and of the internal control during the PCR. In their native state, the probes adopt a folded state which positions the quencher near the fluorescent dye. In this condition, most of the fluorescence of the tracer is absorbed by the near quencher, thus minimizing the fluorescence emitted. When the amplified are generated, the fluorescent marked probes unfold, they hybridize with the new amplified DNA, and so they separate the fluorescent tracer from its quencher and develop a strong emission of fluorescence. During the extension phase, some of the hybridized probes are cut by the exonuclease activity of the polymerase and are released in solution, so their brightness continues to be detectable. The combined fluorescence of both cut and linked probes correlates with the amount of amplified generated, and it is proportional to the amount of DNA of C. trachomatis in the sample. At each cycle, the luminescence of each sample during the exponential phase of PCR is detected.

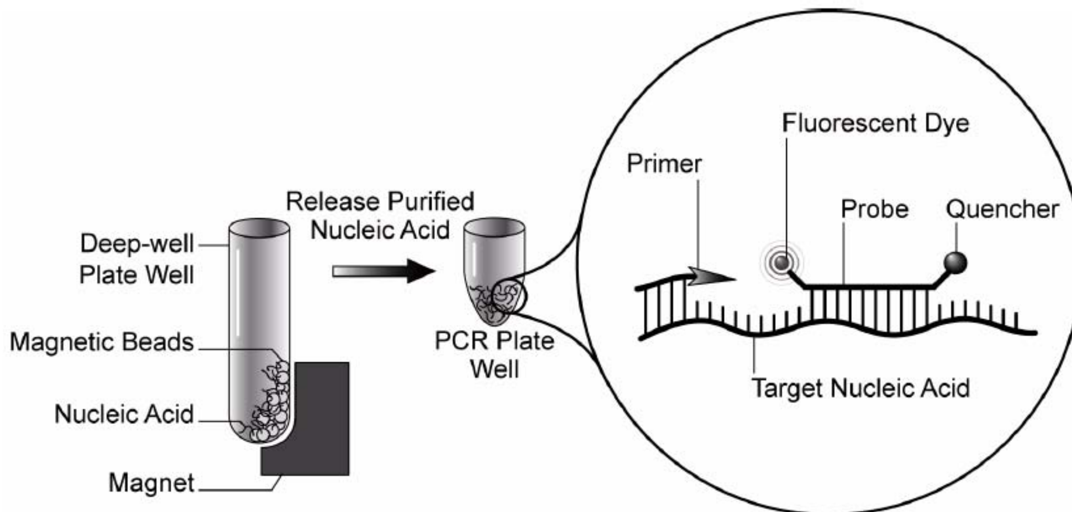

Figure 10. VERSANT kPCR scheme.

Finally, the computer processes the data of the fluorescence for each sample providing an amplification curve and, in case of positivity, the output cycle. According to what has been observed so far, cycle of output appears inversely proportional to the amount of starting DNA (the higher the output cycle, the smaller the amount of starting DNA). At the end of the PCR, the amplification of the internal control, which is designed to get out during the last cycles, must be obtained for each sample.

Typing C. trachomatis with Home-made PCR and RFLP.

The epitopes that characterize different serotypes of C. trachomatis mainly reside on MOMP. The gene encoding for MOMP, called *omp1* or OmpA, is the main target of the genotyping process, analyzing the changes of the gene sequences within the different strains [100].

The *omp1* gene is amplified by the extracts deriving from the residual on the extraction plate of the module VERSANT® kPCR after the session of diagnostic commercial PCR. These extracts are submitted to a semi-nested PCR arranged according to the literature [101,102].

This method consists of a **first session of PCR** in which a fragment of 1033 bp DNA *omp1* gene is amplified, using the primers SERO1A (constituted by the base sequence 5'-

ATGAAAAACTCTGAAATCGG-3') and SERO2A (sequence 5'-TTTCTAGAT/CTTCATT/CTTGTT-3'). These primers are both specific for Chlamydia and able to recognize all strains of the bacterium, binding to the complementary regions of the gene *omp1*. The presence of an amplified of 1033 bases of the gene *omp1* is an index of positivity, since the sequence bound by the primers is specific for this bacterium. For this amplification step, 15 µl of extract were added to a final volume of 50 µl of the reaction mixture, containing:

- 1.6 µl of primer SERO1A 10 µM
- 1.6 µl of primer SERO2A 10 µM
- 10 µl of buffer 5×
- 2 µl DMSO
- 5 µl of dNTPs 2 mM
- 0.4 µl of Taq polymerase (5U/µl)
- 2 $MgCl_2$ 25 mM
- 12.4 µl of H_2O

PCR amplification is carried out as described below:

- 7 min at 94º C (pre-activation of the Taq polymerase)
- 1 min of denaturation at 94º C
- 3 min of annealing at 45° C } 40 cycles
- 3 min of elongation at 72º C
- 7 min at 72º C for an additional extension

The **second session of PCR** has as target the same amplified obtained from the previous procedure, and not the extracts from the commercial kit, thus increasing the sensitivity of the entire method, giving the possibility of genotyping biological samples containing a low charge of bacteria. The conditions for amplification are identical to those previously described, but the DNA fragment used as target of the second PCR is 55 bp shorter than the 1033 bp fragment of the previous procedure. In fact, a different primer pair (one nested primer,pCTM3, sequence 5'-TCCTTGCAAGCTCTGCCTGTGGGGAATCCT-3' and SERO1A) is used compared to the first session; this second primer pair binds the amplified DNA a little more inside, slightly shortening it.

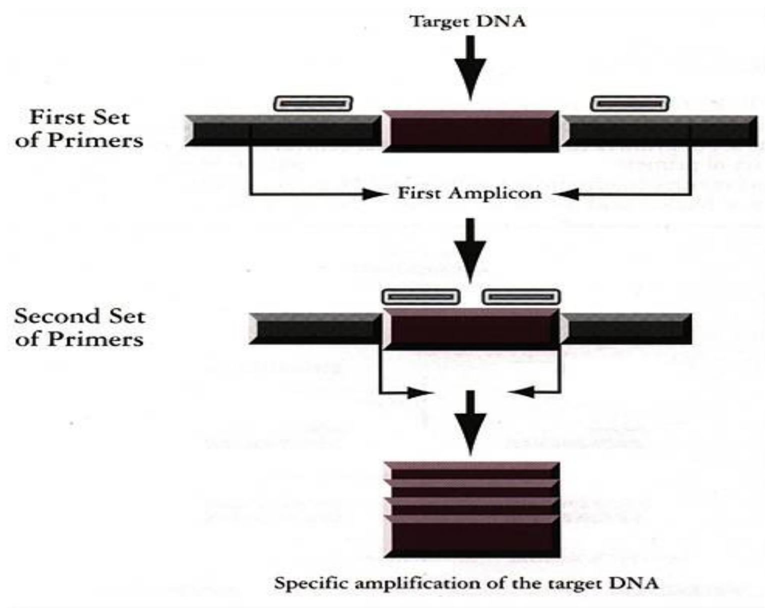

Figure 11. Nested-PCR.

This technique is a semi-nested PCR rather than a true nested PCR, because the two sessions differ only for a primer and not for both (Figure 11).

The reaction mixture of the second session of PCR contains 5 µL of target DNA unlike 15 µl of the previous session, and therefore different amounts of H_2O to bring the final volume to 50 µl, as described below:

- 1.6 µl of primer PCTM3 10 µM
- 1.6 µl of primer SERO2A 10 µM
- 10 µl of buffer 5×
- 2 µl DMSO
- 5 µl of dNTPs 2 mM
- 0.4 µl of Taq polymerase (5U/µl)
- 2 $MgCl_2$ 25 mM
- 22.4 µl of H_2O

Genotyping continues with the use of **Restriction Fragment Length Polymorphism (RFLP)** which exploits the capacity of restriction enzymes to 'recognize' and cut the *omp1* gene previously amplified in a specific way depending on the different bacterial strain.

In order to ensure further validity to the method, the amplified obtained by nested PCR is detected using the electrophoretic run on agarose gel at 1.5% (in particular 1.5 g of agarose in 100ml of buffer 0.5 X TAE), before digestion by restriction enzymes, thus confirming the presence of amplified genetic material, in order to endorse the proper execution of both molecular methods.

RFLP provides the digestion of 10 µl of the nested PCR product with 2.5 U of restriction enzyme AluI (Promega, Madison, WI); if the action of digestion of AluI is not sufficient, or if it is not possible to recognize different strains with the sole activity of this enzyme, restriction enzymes DdeI, HinfI and EcoRI can be used, evaluating their activity on part of the amplified previously stored.

The conditions of digestion can be summarized as follows:

- 10 µl of amplified
- 0.25 µl of AluI (2.5 U)
- 2 µl of buffer 10x
- 0.2 µl of BSA 100x
- 7.5 µl of H_2O

} digestion over night at 37 º C

The product of digestion is subsequently examined by analyzing its electrophoretic run for 45 minutes at 100 V on 12% polyacrylamide gel stained with ethidium bromide. According to the weight and the number of different fragments generated by cutting, the patterns of restriction, and therefore C. trachomatis genotypes, are identified (Figure 12,13,14).

Figure 12. Restriction patterns on polyacrylamide gel after enzymatic digestion.

Figure 13. The DNA stained with ethidium bromide emits a red-orange fluorescence when subjected to UV light.

Figure 14. Restriction patterns resulting from the action of the enzymatic digestion for each C. trachomatis serovar, according to the use of different restriction enzymes (AluI, DdeI, HinfI and EcoRI).

AluI

A	B	Ba	C	D	Da	E	F	G	H	I	Ia	J	Ja	K	L1	L2	L2a	L3
486	244	265	442	241	239	241	321	321	458	458	458	435	442	486	241	227	227	458
295	225	204	241	225	225	225	256	207	298	295	298	298	298	213	228	225	225	298
138	195	195	217	195	195	132	207	193	138	138	138	138	138	138	204	132	131	138
46	89	150	57	68	68	102	101	107	46	46	46	46	46	71	68	102	101	46
33	68	88	28	61	60	96	73	96	33	33	33	44	33	46	61	96	96	33
11	61	68	16	56	56	68	33	73	28	28	28	33	28	33	56	59	59	28
10	56	33	11	42	42	56	10	10	11	11	11	11	16	14	42	56	56	11
	33	10	10	41	41	42	9	9	10	10	10	10	11	11	41	48	48	10
	32			33	33	17	6					7	10	10	21	17	17	
	10			32	32	15									17	15	17	
				10	10	10									15	10	15	
					6										10		10	
															6		9	

DdeI

A	B	Ba	C	D	Da	E	F	G	H	I	Ia	J	Ja	K	L1	L2	L2a	L3
323	293	293	414	435	434	369	605	605	414	323	323	414	414	414	320	293	193	414
231	251	251	323	293	292	293	411	385	323	231	231	323	323	288	293	164	164	323
183	187	187	153	282	281	184		26	153	183	183	153	153	153	282	150	150	153
150	150	150	132			164			132	149	153	132	132	132	115	132	131	132
132	132	132								132	132			35		115	115	
																87	87	
																72	72	

HinfI

A	B	Ba	C	D	Da	E	F	G	H	I	Ia	J	Ja	K	L1	L2	L2a	L3
530	591	591	998	392	391	588	764	817	533	529	533	533	533	557	588	591	590	533
465	237	238	24	237	236	237	175	175	465	465	465	441	465	441	237	237	237	465
24	161	161		196	195	161	53	24	24	24	24	24x2	24	24	161	161	161	24
	24	24		161	161	24	24								24	24	24	
				24	24													

EcoRI

A	B	Ba	C	D	Da	E	F	G	H	I	Ia	J	Ja	K	L1	L2	L2a	L3
963	826	826	963	823	820	328	960	629	966	962	965	966	966	966	823	498	498	835
56	131	131	56	131	131	269	56	331	56	56	56	56	56	56	131	328	327	131
	56	56		56	56	226		56							56	131	131	56
						131										56	56	
						56												

Results.

During the study, 84 anorectal swabs for CT/GC PCR were performed on 6 women and 78 men. VERSANT® CT/GC DNA 1.0 Assay identified 14 positive samples for C. trachomatis; typing with semi-nested PCR and RFLP identified 6 serovars L2 in 5 patients (one of the patients reported 2 different infections by C. trachomatis serovar L2), 2 serovar E and 1 serovar J. It was not possible to typify 5 of the positive samples because of the low presence of chlamydial DNA in the sample itself.

The patient with CT serovar J infection was positive also for GC infection. All positive patients were MSM.

Features and clinical history of the 5 patients with LGV proctitis are summarized below (Figure 15,16,17).

Patients	Anal ulceration	Anal pain	Anal discharge	Constipation Tenesmus	Inguinal lymphadenophaty	First consultation	Diagnostic hypothesis
1	x	x	X	X	x	STDs clinic	Syphilis CT/NG proctitis Trauma
2	x	x		X	x	Primary care physician	HSV
3	x	x	X	X		Primary care physician	Enteritis
4	x	x	X			Primary care physician	IBD CMV Solitary ulcer
5		x	X	X		Primary care physician	IBD

Patients	Proposed examination	Results	First treatment	Outcome
1	Screening STDs Anal swabs for CT/NG Urine for CT/NG	Rectal swabs +	Doxycyclina	Healing
2	Abdomen US	Monolateral inguinal lymphadenopathy	Brivudine/ acyclovir	No improved
3	Endoscopy	Internal hemorroids	Ciprofloxacin	Slight improved
4	Endoscopy (colonscopy)	Anal and sigma mucosal ulceration	Mesalazine/ prednisolone	Slight improved
5	Proctosigmoidoscopy Transanal US	Rectal warts Internal hemorroids	Surgery/ diosmin	No improved

Figure 15. Summary of clinical symptoms and diagnostic pathway of the patients.

Patients	Age	Sexual behavior	N°partner last 6 mo.	Condom	Other STDs	HIV	Viral load	CD4+	CD4/CD8
1	39	bisex	6	Sometimes	Syphilis GC urethritis HBV				
2	42	MSM	>10	No	Syphilis Perianal warts HSV/ HBV	x	<20copies/ml	834	0,71
3	43	MSM	>5	No	Syphilis	x	<20copies/ml	859	0,29
4	48	MSM	1	No	Syphilis	x	<20copies/ml	549	0,65
5	44	MSM	¾	Sometimes	Anal warts				

Figure 16. Summary of sexual behavior and co-infection with other STDs.

Patients	PCR	Exit cycle	Time for diagnosis	Antibodies U/mL	Treatment	Outcome	Time for clinical healing	PCR after 3 months	Antibodies U/mL after 3months	Only if symptoms recur	
										Rectal PCR	**Antibodies U/mL**
1	Rectal swabs+ Urine -	31,5	1 month	IgA 7 IgG 39	Doxycycline 100 mg x2/die 21 days	Healing	~10 days	CT-LGV neg CT/GC pos	IgA 6 IgG 57		
2	Rectal swabs+ Urine-	21,62	2 months	IgA 12 IgG 18	Doxycycline 100 mg x2/die 21 days	Healing	~10 days	CT-LGV neg	IgA 8 IgG 19	CT-LGV pos NG pos (after 9 months)	IgA 7 IgG 37 (after 9 months)
3	Rectal swabs+ Urine-	32,83	1 months	IgA 42 IgG 52	Doxycycline 100 mg x2/die 21 days	Healing	~15 days	CT-LGV neg	IgA 37 IgG 58		
4	Rectal swabs+ Urine-	26,41	11 months	IgA 8 IgG 86	Doxycycline 100 mg x2/die 21 days	Healing	~12 days	CT-LGV neg	IgA 5 IgG 112		
5	Rectal swabs+ Urine-	25,7	23 months	IgA 8 IgG 60	Doxycycline 100 mg x2/die 21 days	Healing	~20 days	CT-LGV neg	IgA 7 IgG 98		

Figure 17. Summary of laboratory results at the time of diagnosis and after treatment.

Clinical presentation.

All patients with LGV proctitis were symptomatic when visiting our Clinic. All of them complained of anal pain, occurred with change in bowel habit, like constipation or tenesmus (4 patients), and mucous or bloody anal discharge (4 patients). During clinical examination anal or perianal ulceration were noticed in 4 patients: they were of small size and their edges were not infiltrated (Figure 18). Only in 2 patients a monolateral, not painful, inguinal lymphadenopathy was appreciable (Figure 19)(Figure 15).

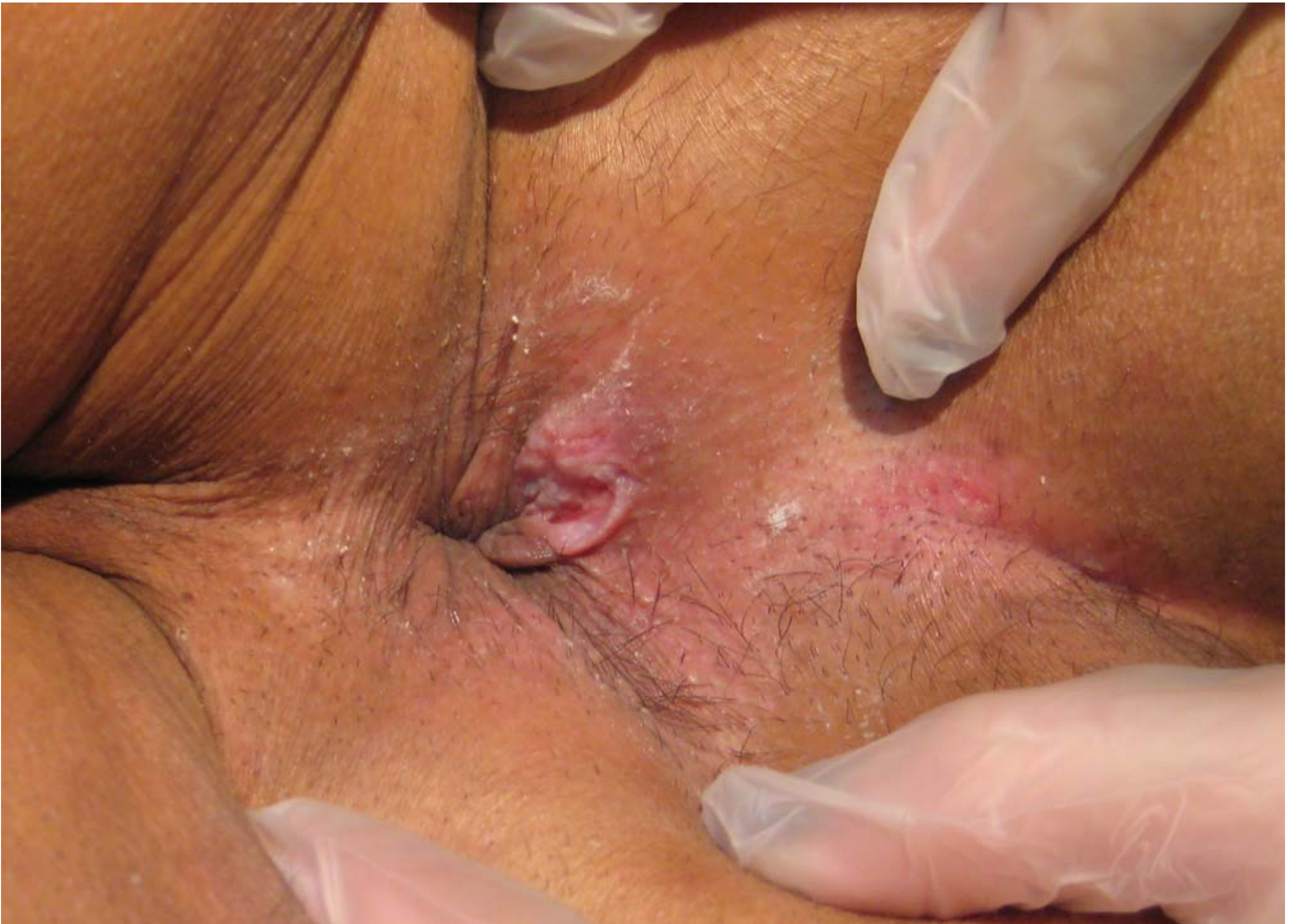

Figure 18. Perianal non infiltrated ulceration in a patient with LGV proctitis.

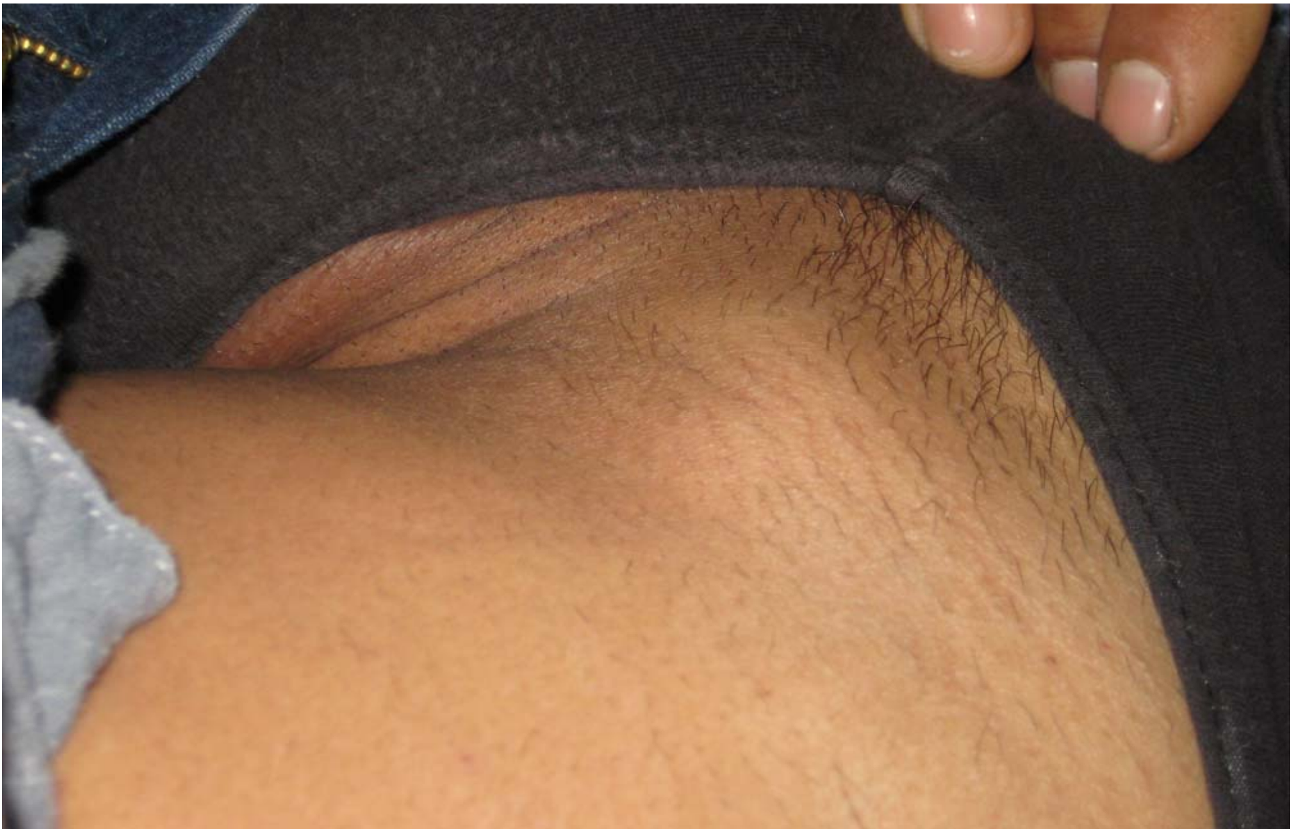

Figure 19. Monolateral inguinal lymphadenopathy in a patient with LGV proctitis.

Recent medical history.

Only one patient (patient n°1) came quickly to our Clinic because he already knew it; after a few weeks, the time necessary for the laboratory results, diagnosis of LGV proctitis was established.

The other patients firstly asked advice to their primary care physician.

Patients n° 2, presenting perianal ulceration and inguinal lymphadenopathy, after undergoing an abdominal ultrasound that confirmed inguinal lymphadenopathy, received a diagnosis of perianal herpes simplex infection and was treated with acyclovir and brivudine without any improvement.

Patients n° 3 underwent a proctological consultation and a rectal endoscopy that revealed only inflammation of the internal hemorrhoidal plexus; he was treated with ciprofloxacin for 6 days with slight improvement and after 1 month he came to our Clinic.

Patient n° 4 complained of anal symptoms for 11 months, before having the right diagnosis. During this time he underwent colonscopy that revealed a widespread recto-sigmoiditis with multiple ulcerations; histological examination of the affected mucosa showed alterations of

the glandular morphology, ulceration of the mucosal surface, cryptitis and lymphocytic infiltrate in the lamina propria, suggesting Crohn's disease. Patients was treated with prednisolone and mesalazine for several weeks without any improvement. A *Cytomegalovirus* reactivation, since patient was HIV-positive, and a solitary rectal ulcer were the subsequent diagnostic hypotheses.

Patients n° 5 reported the beginning of anal symptoms in July 2010; during the 23 months from the onset of the symptoms to diagnosis, he underwent proctosigmoidoscopy that revealed inflammation of internal hemorrhoidal plexus and rectal warts. Warts were treated surgically; inflammation of hemorrhoids did not improve with several cycles of oral diosmin. A following proctosigmoidoscopy showed wound healing difficulty in the seats of previous surgery (Figure 15).

Risk factors.

The age of patients ranged from 39 to 48 years old; the mean age was 43,2 years.

Four patients were MSM, while one patient declared to be bisexual; all of them reported receptive anal intercourses in the last 6 months. Three patients declared they never used condom, while 2 patients used condom rarely .

Three patients remembered more than 5 sexual partners in the last 6 months; patient n° 4 reported a stable relationship with only one partner.

All patients suffered from other STDs in the past. Four of them presented a positive serology for syphilis (2 with early latent syphilis and 2 with previously treated syphilis); 2 patients reported a positive anamnesis for perianal or anal warts; 2 had hepatitis B; one had herpes virus genital infection and one a previous episode of gonococcal urethritis.

A few months after diagnosis and treatment of LGV, two patients reported a new sexually transmitted infection: patient n°1 was affected by proctitis caused by C. trachomatis non-L and N. gonorrhoeae 3 months after, while patient n° 2 presented a new LGV infection 9 months after the first episode.

Three patients were HIV-positive. All of them had a well-controlled infection with viral load < 20 RNA copies/mL; two patients underwent a treatment with HAART (Figure 16).

Laboratory data and outcome.

All patients with LGV proctitis had a positive rectal swab for C. trachomatis serovar L2; CT/NG PCR on/in urine was negative. The PCR exit cycle was relatively low for patients n° 2, 4 and 5; it indicated that the starting material contained abundant chlamydial DNA. In patients n° 1 and 3 the exit cycle was high, indicating a low presence of bacterial DNA in the swabs; it could be due to the time and modality used in the collection of the sample.

All patients were treated with doxycicline 100 mg twice a day for 21 days; all of them completed the treatment without side effects. Clinical symptoms and signs completely recovered during treatment. After 3 months the rectal swab for CT/NG PCR was negative in all patients, except patient n°1 who resulted positive for N. gonorrhoeae and C. trachomatis serovar non-L proctitis, with recurrence of mild anal symptoms (mucous discharge). Patient n°2 came back to our Clinic after 9 months from the first diagnosis of LGV because of recurrence of anal ulceration and pain; a new rectal swab was positive for N. gonorrhoeae and C. trachomatis serovar L2, witnessing a LGV re-infection.

Serum antibodies against Chlamydia were positive in all patients, with very different values; IgA ranged from 7 to 42 U/mL, while IgG ranged from 18 to 86. At the control visit after 3 months, IgA slightly decreased and IgG were stable or slightly increased (Figure 17).

Discussion.

In the last decade, a new outbreak of LGV proctitis mainly in MSM has been registered in the industrialized countries. LGV recurrence represents a public health problem, as its ulcerative nature may facilitate transmission of HIV and other STDs [67]. Diagnosis of LGV is not easy and requires specific diagnostic test not available in all laboratories; therefore, LGV is often misdiagnosed by primary care physicians and doctors who do not deal daily with STDs. Moreover, in many countries there is no effective surveillance system for this disease and data about epidemiology are uncertain. This re-emerging disease requires greater attention and resources.

Since January 2012, patients attending our STDs Clinic for anorectal symptoms or simply with a positive anamnesis for unprotected receptive anal intercourse in the last 6 months have been proposed to carry out a clinical visit, an anorectal swab and a sample of urine for detection of C. trachomatis and N. gonorrhoeae infections using a Real-Time PCR. Positive

samples for C. trachomatis underwent genotyping; in this way 5 cases of LGV proctitis, and a re-infection in one of the patients, were diagnosed.

Prevalence of LGV in the group of tested patients is 7,1%. It must be considered that we screened a high risk population where most of the patients complained of anorectal symptoms and so they were more motivated than others to undergo clinical visit and rectal swab. Our purpose was not to develop an epidemiological study to determine incidence and prevalence of LGV. In this case the study should have been performed on a larger population both symptomatic and asymptomatic; it is not easy to propose a rectal swabs as a screening test, partly because this exam assumes that the patient had passive anal intercourse. Some MSM may avoid seeking healthcare because they expect disapproval regarding their sexual behavior and the sanitary staff is not always able to ask behavioral screening questions in a professional manner, without implicit assumptions [103]. Therefore, statistical prevalence of LGV in our samples cannot be considered a pure epidemiological value and it is difficult to compare it with other works that reported a prevalence value of about 1% in MSM patients attending a genitourinary clinics [38,39].

The mean age of our patients was 43,2 years, in line with the epidemiologic data of ECDC that described the greatest number of cases in the age category 35-44 years old, followed by the 25-34 years old. By contrast, the incidence of C. trachomatis non-L infection is higher in the age group 20-24 years old [37].

Our cases of LGV infections presented in MSM or bisexual men, and this is in line with European data [37]. In fact, in all industrialized countries, LGV proctitis has been described almost exclusively in MSM and the detection of LGV in the general population has not been confirmed up to now [63], if not for some sporadic cases [54,55].

The majority of patients reported high-risk sexual behavior including a high number of sexual partners, unprotected anal intercourse, fisting, sharing sex toys, behaviors often connected to a sex party scene and involving international sexual networks. In a cross-sectional study the use of enemas was found to be associated with a higher risk of LGV

proctitis, probably because this procedure leads to mucosal damage before receptive anal intercourse favoring the transmission of the disease [67]. Information about sexual behavior in our patients only concerns the use of condom, that is never or only sometimes used by 5 men previously described, and the number of sexual partners in the last 6 months. Four patients reported they have had multiple sexual partners, although the number vary from case to case, while patient n°4 reports a stable relationship for years.
He and his partner reported unprotected sex with each other, passive to our patient, who is HIV-positive, and active for his partner, and both denied having had sexual relations outside the couple. The serological evaluation of the partner, HIV negative, showed an antibody titre equal to 8 U / ml for IgA and 110 U / ml for IgG, suggesting a previous contact with the bacterium. However, the lack of a rectal swabs and urine sample for PCR does not allow us nothing more than a hypothesis, also because the partner was asymptomatic throughout the period of study. Evaluating the information provided by the couple, the presence of a high antibody titre and the lack of appearance in the partner may suppose a case of urethral asymptomatic LGV in the partner, unknowingly transmitted to our patient.
It is also interesting to note that patient n°5 reported a relatively low number of partners during the six months preceding the infection and, although he referred a sporadic use of condom, screening for other STDs was negative, except anal warts. Some rare cases of LGV in HIV-negative patients who claim a few sexual contacts are reported in literature [53].
Finally, the clinical history of patient n° 2 should be underlined. In fact, he reported a clinical and laboratory healing after treatment with doxycycline, but after about 9 months he came back to our Clinic because of a recurrence of rectal symptoms and a new diagnosis of LGV proctitis was made. He admitted a new unprotected anal intercourse with one of the previous partners who, although informed of the disease, chose not to undergo medical check-up and not to take the antibiotic treatment. This behavior underlines the tendency to underestimate the risk of acquiring STDs and their possible complications in some groups, as HIV-positive MSM.

Three of our patients (60%) are HIV-positive. The HIV infection has been identified as the strongest risk factor for anorectal LGV [66] and percentage of co-infection ranges from 67% to 100% across the reports [68]. MSM have not been confirmed to be a reservoir of LGV

and the high rate of HIV co-infection probably reflects the confounding effect of HIV as a surrogate marker of high risk behavior. Nevertheless, a potential effect of HIV infection, increasing the susceptibility to infection or facilitating the expression of the disease, cannot be ruled out. Since the introduction of highly active anti-retroviral therapy, an increase of unprotected sexual practices has been observed worldwide among both HIV -positive and HIV-negative MSM; this fact could have contributed to the spread of LGV among MSM [67]. In the recent outbreak, the majority of patients were HIV-positive with preserved CD4+ cells count, as it is for our patients. Clinical features, answer to treatment and outcome of LGV appear to be similar regardless of concomitant HAART, of the count of CD4+ or of the viral load [59,66] and therapy with doxycycline for 21 days seems to be equally effective both in HIV-negative and -positive patients [37].

On the other hand, the ulcerative and inflammatory nature of LGV could enhance transmission and acquisition of HIV and other STDs; in fact, also the presence of a non-ulcerative infection may facilitate the infection by HIV through an increase in local inflammatory cells, which are the target of this virus [104]. Four of our patients (80%) have a positive serology for syphilis, 2 of them diagnosed as early latent syphilis. The association between HIV and syphilis has been described previously in several works [105-108]; it may be supposed that HIV, syphilis and LGV essentially share common risk behaviors.

On this topic, we should remember two important concepts that may explain the low use of condoms by HIV positive subjects and the increased incidence of STDs and risky sexual behavior among MSM since the late Nineties. Firstly, the risk of infection depends upon the kind of microorganism and sexual practice: for example, unprotected oral sex is associated with a low risk of transmission of HIV and a relatively high risk of transmission of other STDs. Secondly, often MSM choose sero-adaptive behaviors so as to reduce the risk of transmission of HIV during unprotected anal intercourse. These behaviors tend to reduce the transmission of HIV without changing the incidence of other STD; in this regard, we define *serosorting* the selection of partners with the same HIV status and *seropositioning* the behavior for which during anal intercourse the HIV-positive partner is always receptive [109,110]. The problem of the increase incidence of other SDTs in HIV-positive MSM is very current; some recent studies observed a significant reduction in STD incidence after implementing routine STD testing together with a brief risk-reduction counseling from

medical providers, offering specific knowledge about non-HIV STDs and their possible complications [111].

In the literature, LGV proctitis is symptomatic in the majority of cases, with percentage ranges from 80% to 100% in the different reports; it occurs in more than 90% of cases with a moderate or severe ulcerative proctitis or proctocolitis [67], unlike proctitis caused by C. trachomatis non-L that in many cases runs silent [81]. The most reported symptoms are rectal pain, anal discharge and rectal bleeding, while functional symptoms are more rarely described.

All our 5 patients were symptomatic and the clinical presentation was similar to those of other reports, except for anal/perianal ulceration and functional symptoms (constipation, tenesmus), both present in 4 of our patients. Anorectal ulceration are rarely reported in literature (4-57% of cases), probably because of their transient nature or of a unique rectal location, easily being unnoticed during a visit of the external genitalia. In fact, in a Dutch study of 2004, several patients whit LGV underwent sigmoidoscopy that revealed ulcers of the rectal ampoule in all of them [112]. Nevertheless, LGV proctitis follows direct infection of the rectal mucosa and an ulcerative transient lesion, usually simultaneous to other symptoms, may be present, but no clear distinction between "primary" or "secondary" proctitis is possible [67,45]. Constipation and tenesmus, probably due to transmural and perirectal inflammation and edema associated with the disease, are described in the literature as less frequent but more specific than LGV proctitis, compared to other infective rectal diseases [73].

Inguinal node enlargement are reported in a low number of cases in the literature, with percentages ranges from 13% [33,59] to 24% [66] and 43% [34]; 2 of our patients present unilateral inguinal lymphadenopathy. This symptom may be linked to the location of the site of infection on perianal skin.

All our LGV cases have anorectal localization and are substantially similar to the cases of the literature. Although patients reported passive and active anal intercourse, an urethral infection with C. trachomatis serovar L has not been identified. This is in line with the general overview reported internationally and emphasizes the problems encountered to identify the reservoir of infection and the actual mode of transmission among MSM. It has

been hypothesized that the transmission of rectal LGV may be related to other sexual activities, such as “fisting” or using sex toys [56,112].

The scientific literature of the last decades is rich in case reports of LGV proctitis initially misdiagnosed with other disease, especially inflammatory bowel disease, since endoscopic appearance of the rectal mucosa and sometimes also histological examination show common characteristics between the two disease [82,113]. In some cases, the use of instrumental tests as endoscopy may delay diagnosis by providing not specific information.
In our work, patients n° 4 and 5, who underwent endoscopic examination, suffered from the biggest delay in diagnosis since endoscopic examination did not lead to the right hypothesis and delayed other investigations. The importance of considering the disturbances reported by patients, rather than the conclusions drawn exclusively from the results of instrumental tests, must be emphasized.
Differential diagnosis with other STDs must be also remembered: syphilis, perianal herpes simplex, C. trachomatis non-L and N. gonorrhoeae proctitis can provide a clinical picture very similar to LGV proctitis and should be excluded by laboratory tests [83].

All 5 patients were treated with doxycycline 100 mg twice a day for 21 days, with complete resolution of clinical symptoms by the end of treatment, regardless of their HIV status [83,114]. Test of cure for rectal C. trachomatis after 3 months did not show any persistence of Chlamydia serovar-L DNA in any patient. Patient n°2, who reported a new LGV proctitis 9 months after the first, was treated again with doxycycline with resolution of clinical symptoms.

LGV proctitis is usually associated with significantly higher titre of IgA and IgG antibodies in comparison with C. trachomatis non-L infection, probably because of more invasive chronic inflammation of the tissue; in fact, LGV serovars are able to leave the host cell at the basolateral surface to infect cells under the epithelial layer up to reach the connective and lymphatic tissues, in contrast to serovars non-L that remain confined to the epithelium. Despite this, IgA and IgG serological titres alone do not allow a *certain* diagnosis of LGV; in case of high titre associated to a positive rectal swab for C. trachomatis in a symptomatic

patient diagnosis of LGV is defined *probable*, while serological high titre in a symptomatic patient, without identification of Chlamydia, makes the diagnosis only *possible* [80,115,116]. On the other hand, a low antibody titre does not exclude LGV infection in a symptomatic patient.

In conclusion, although serology is often positive in LGV cases, it is not a suitable method for screening and diagnosis, if biovar L specific NAAT is available [116]. All our patients present a positivity for IgA and IgG serology with different values which range from titres just above the cut-off index to high titres. This variability does not seem to be correlated to the HIV state or to the diagnostic delay.

IgA and IgG antibodies are not really useful even in follow up after treatment, at least during the first year. Although IgA reactivity tends to decline after the acute infection, it usually does not fall under the cut-off value; the decrease of the IgG titer is usually still slower over time. Consequently, IgA and IgG antibodies are not suitable to differentiate past effectively treated infection from successive re-infections [117]. This trend of antibodies titres is confirmed in our patients; in fact, at the control visit after 3 months, IgA slightly decreased in all patients but they are still positive according to our laboratory cut-off and IgG are stable or slightly increased.

A certain diagnosis of LGV must be supported by identification of C. trachomatis biovar L.

In a comparative study between the cell culture and NAATs (nucleic acid amplification testing) for diagnosis of infections by C. trachomatis was found that the NAATs are significantly more sensitive than culture and they can be considered the new "gold standard" for laboratory diagnosis of Chlamydia, both for genital and non-genital infections [118]. In particular, the use of Real-Time PCR for diagnosis of infection with C. trachomatis from rectal swabs increases up to 100% the possibility of identifying the infection compared to culture method [119].

The major defect of the Real-Time PCR used in our work regards the possibility of a genetic mutation of C. trachomatis that could render ineffective its ability in identifying and therefore amplifying the DNA of the microorganism. This potential situation was widely described in the literature in 2006, when a new variant of C. trachomatis serotype belonging to E, with a 377-bp deletion in the cryptic plasmid, was described in Sweden, where a high

percentage (10-65%) of infected patients were reported; nowadays there is currently no evidence that the variant has spread widely throughout Europe. This new variant cannot be detected by amplification of genomic segment deleted, but can be detected by amplification, for example, of the chromosomal gene *omp1*.
All patients positive for rectal C. trachomatis with Real-Time PCR, have been submitted to a Home-made PCR targeting *omp1* and subsequently to RFLP. Home-made PCR is well described in the literature as a quite rapid, economical and extremely reliable technique [101,102]. PCR and RFLP are still abundantly used, especially for the evaluation of samples from patients with LGV [119,120]. The main defects of this method are the inability to detect punctiform mutations in the genoma of the microorganism and to identify the sexual network to which a certain serotype belongs. However, at least the latter restriction does not invalidate the results of our study because sexual networks assessment is outside our purposes.

The results of our work do not provide sufficient evidence to recommend routine screening by anorectal samples for all MSM but, in line with the European recommendations, they confirm, the indication to perform the PCR diagnostic test on rectal swabs and the serotyping, in presence of ulcerative proctitis in MSM or in sexual contacts of subjects affected by LGV [56,83]. However, even considering the limited number of patients examined in our study, the strong association found between LGV and HIV infection suggests that screening for LGV may be recommended in all HIV-positive MSM, as already proposed in other studies [33].

Conclusions.

Since 2003, a new outbreak of LGV, mainly with rectal localization, has been spreading among MSM in Western Europe, the United States and Australia. LGV proctitis typically presents as anorectal syndrome caused by C. trachomatis serovar L2 in most of the cases; the main risk factors are HIV positivity and high risk sexual behavior. Well definite data about its prevalence are not available; LGV is not subject to mandatory reporting in most of the countries and epidemiological surveillance is active only in some countries and it often provides data about high-risk population only. Moreover, a certain diagnosis is based on a diagnostic test which is not available in every laboratory.

This disease is certainly present also among the Italian population, but its true prevalence is unknown. To our knowledge, less than 20 cases of LGV proctitis have been published in Italy until today [121,122,123]; the new outbreak of LGV proctitis is still little known among the medical staff who does not deal routinely with STDs.

With the present study, we identified and treated 6 cases of LGV proctitis in 5 patients. Epidemiological and clinical data and risk factors are broadly in line with the international literature.

With our work, we aim at emphasizing the importance of remember the existence of LGV proctitis, especially when we deal with MSM patients complaining of an anorectal syndrome; moreover we are interested in reaffirming the importance of asking and considering information about the patient's sexual behavior and the need to address the patient to a Center able to make a laboratory diagnosis of certainty with C. trachomatis NAATs and serovar typing.

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