Seroprevalence of Some Bacterial Sexually Transmitted Infections in Patient Groups of Special Epidemiological Relevance in Hungary

Ph.D Thesis

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CONTENTS

ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
COI	cut-off index
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme Linked Immunosorbent Assay
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
IBD	Irritable Bowel Syndrome
Ig	immunoglobulin
IUSTI	The International Union against Sexually Transmitted Infections
kDa	kiloDalton
LGV	lymphogranuloma venereum
LPS	lipopolysaccharide
MIF	micro-immunofluorescence
MOMP	Major Outer Membrane Protein
MSM	Men Who Have Sex with Men
NAAT	Nucleic Acid Amplification Test
OD	Optical Density
OEK	Országos Epidemiológiai Központ
OKI	Országos Közegészségügyi Intézet
ON	Ophthalmia Neonatorum
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PID	Pelvic Inflammatory Disease
RPR	Rapid Plasma Reagin
RSV	Respiratory Syncytial Virus
RTI	Respiratory Tract Infection
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection
TPHA	*Treponema pallidum* Haemagglutination Assay
VDRL	Venereal Disease Research Laboratory
WHO	World Health Organization

1. INTRODUCTION

1.1. BACTERIAL SEXUALLY TRANSMITTED INFECTIONS (STIs): MAIN PATHOGENS, EPIDEMIOLOGICAL DATA

Based on recent WHO data the most frequent bacterial agents with a proven track record of causing venereological diseases (or sexually transmitted infections, STIs), i.e. *Chlamydia trachomatis* (*C. trachomatis), Neisseria gonorrhoeae (N. gonorrhoeae)* and *Treponema pallidum (T. pallidum)* are responsible globally for about 220 million acute infections per year, such as urogenital chlamydiasis, gonorrhoea, syphilis and lymphogranuloma venereum [1]. Based on the records of the national epidemiological reporting system (OSZIR: Országos Szociális Információs Rendszer) the incidence of these infections in Hungary are in harmony with the global STI-trends; and their numbers have reflected an increasing tendency in the last decades. The collated nationwide data of 2017 show that altogether 714 acute and past syphilis, 1176 acute gonorrhoeal and 882 acute urogenital chlamydial cases were diagnosed, which may still underrepresent the current epidemiological situation of Hungary [2].

It must be taken into account that, besides genital contact, oral and anal sexual contacts must also have a significant role in the horizontal transmission of STIs among sexually active people, which must be taken into consideration not only in the case of homo- and bisexual patients but also in that of heterosexual ones. As these infections may have systematic as well as extragenital clinical manifestations due to the above mentioned various transmission routes, dermato-venereologists and other physicians representing almost every medical specialty can face the often challenging symptoms of STIs, which also frequently raises the problem of differential diagnostics. However, one must focus not only on STIs among adults but also on neonatal STIs caused by a vertical transmission indicating the infection of the mother and of her sexual partner(s) and pointing to the need for the involvement of other clinicians (gynaecologists, urologists, etc.) to diagnose and treat all of them.

Besides active prevention, one of the most important prerequisites of fighting off the spread of STIs is the early diagnosis thereof together with their adequate treatment and contact tracing. Albeit there are lots of problems hampering this effort, such as the patients' fear of prejudice and the feeling of being stigmatised, in addition to the wanting knowledge regarding relevant sampling, let alone certain pitfalls of laboratory diagnostics.

The laboratory diagnosis of bacterial STIs should focus on the direct detection of pathogens in the case of local infections, while the more invasive and/or systematically manifesting venereological diseases can be tested with serological tests. The detection of the specific immune response triggered by *C. trachomatis* L serotypes or *T. pallidum* serves as a reliable diagnostic option [3,4].

In my thesis I have aimed to analyse the current epidemiological data focusing on the latter STIs, achieved with modern serological methods in three study groups of special relevance: neonates, MSM (men who have sex with men) and pregnant women in Hungary.

1.2. *C. TRACHOMATIS* INFECTIONS

1.2.1. Urogenital infections in adults caused by D-K serotypes

C. trachomatis infections due to D-K serotypes remain the most frequently reported bacterial STIs in the world [1]. The majority of acute chlamydial infections (typically urethritis, cervicitis, proctitis) have an asymptomatic course, in as many as 90% of women and almost in 50% of men [3]. As a consequence, infected, untreated men may suffer from an epididymitis, while the auto- or heteroinoculation of the contaminated genital secretion can cause conjunctivitis in both genders as an extragenital manifestation of this STI [3,5]. Heterosexual transmission accounts for a high rate, approximately for 90% of the cases [6]. The ascending acute urogenital infection may cause a severe, chronic pelvic inflammation (manifesting as endometritis, salpingitis, pelvic inflammatory disease [PID], chronic pelvic pain etc.) in 15-40% of untreated women, with a risk of consequent infertility and ectopic pregnancy due to tubal damage [7]. In contrast to the non-complicated uro-/anogenital *C. trachomatis* infections these ascending, chronic type of infections induce a systematic immune response, which enables the etiological investigation of this group of patients with serological methods [8].

1.2.1.1. Relevance and prevention of vertical transmission

The vertical transmission of *C. trachomatis* in pregnant women with cervicitis threatens their neonates, who may be infected most often via contact route, in the contaminated birth canal. Based on literature data the risk of transmission is between 50 and 70% [9]. The most frequent manifestation of neonatal chlamydial infection is a local inflammation of the

conjunctivas, called ophthalmia neonatorum (ON), which develops in 30-50% of the infected neonates. The onset of the invasive infection of the respiratory tract occurs at a lower rate, between 10-20% of them. (**Figure 1.**) This subacute pneumonia is caused by the aspiration of the contaminated nasopharyngeal secretion and approximately half of the respiratory tract infection (RTI) cases are accompanied with the telltale sign of ON [10,11,12].

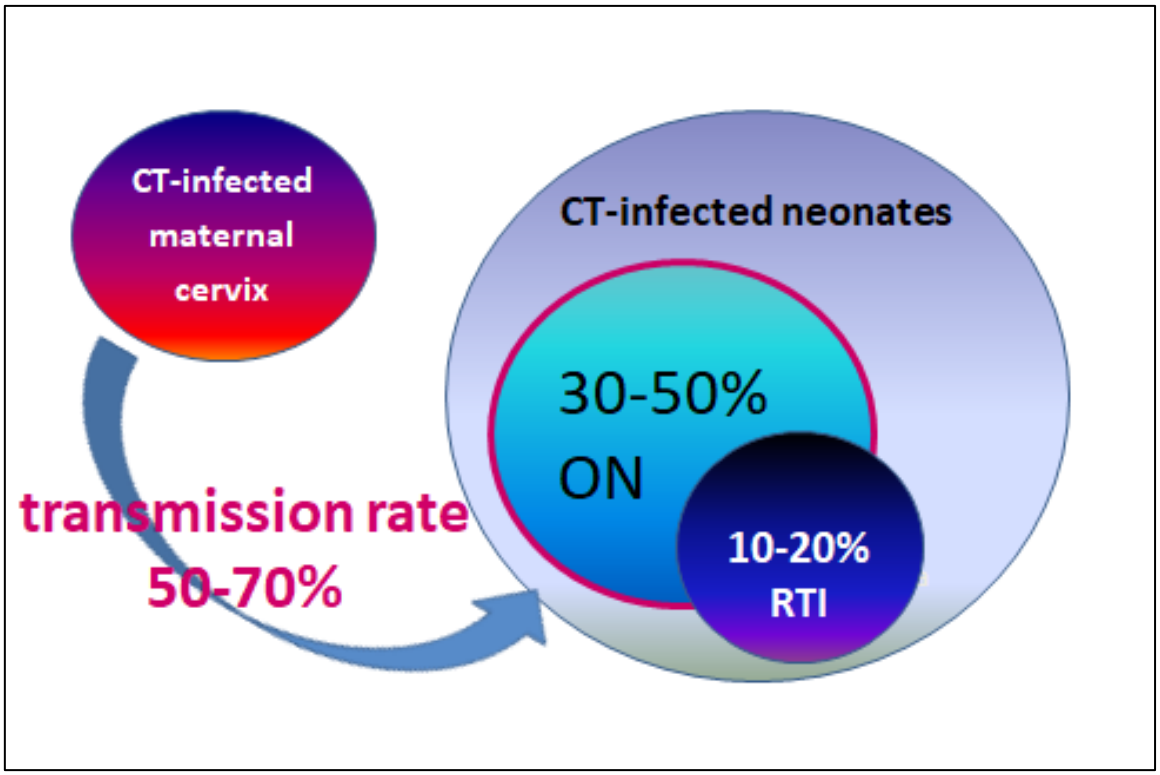

Figure 1. Rate of vertical transmission of *C. trachomatis* and manifestation of infection among colonised neonates [9]

(abbreviations: CT- *Chlamydia trachomatis*, ON - ophthalmia neonatorum, RTI - respiratory tract infection)

Pertussis, as a clinically similar RTI, may arise as a differential diagnostical problem for neonatologists, particularly because chlamydial pneumonia is a so-called „late-type" infection, affecting mainly the age group of 4-12 week old neonates, who are partially unvaccinated against pertussis [13].

The routine prenatal screening in Hungary currently does not include *C. trachomatis* maternal cervical tests. However, in accordance with the latest recommendations of Centers for Disease Control and Prevention (CDC), all pregnant women aged <25 years and those at an increased risk for chlamydia (e.g., those who have a new sex partner, more than one sex partner, a sex partner with concurrent partners, or a sex partner who has a sexually transmitted infection)

should be screened not only at first prenatal visit but should be also rescreened during the third trimester [14].

Due to the lack of a routine antenatal *C. trachomatis* screening in Hungary we have no prevalence data among pregnant women from which we could deduce the actual risk of neonatal infections. The majority of neonatal *C. trachomatis* infections are most likely misdiagnosed and unrecognized conditions as only targeted laboratory investigations could clarify their etiology. The lack of etiological identification and that of consequently reported data make it even more difficult to assess the actual prevalence of chlamydial infections among infants. A generalized screening approach of pregnant women would be a crucial tool of prevention completed with the adequate treatment and follow-up aimed to reduce the number of infected mothers and thereby the rate of vertical transmission, not to mention the potential maternal sequelae of untreated cervicitis.

1.2.1.2. Diagnostic relevance of the systemic immune response in neonates

Similarly to adults' uro-/anogenital infections the local inflammation in ON, i.e. the conjunctival form of neonatal *C. trachomatis* infections does not induce any systemic immune response, which means that serological tests are not useful to diagnose these conditions.

The more invasive type of *C. trachomatis* infections, i. e. RTI elicits a specific IgM response in neonates, which can be detected for diagnostic purposes. As the maternal IgM does not pass across the placenta, the presence of *C. trachomatis* specific IgM antibody in newborns' blood samples is indicative exclusively of a systemic immune response of the neonates. When detected in a cut-off (or higher) dilution recommended by international guidelines, it is a valuable diagnostic tool in supporting the chlamydial etiology [3,15].

1.2.1.3. Possibilities of laboratory diagnostics in neonatal infections

The gold standard for the laboratory diagnosis of neonatal *C. trachomatis* infections regarding both ON and RTI is the direct detection of pathogen from relevant clinical samples. Conjunctival scrapings or nasopharyngeal aspirates (serving as a reservoir of a more invasive, descending RTI) are recommended to test for the presence of *C. trachomatis.* In serious cases of hospitalized infants the investigation of a low respiratory tract specimen (such as tracheal aspirate, bronchoalveolar lavage or lung biopsy tissue) could also be an alternative [14].

For the testing of the chlamydial DNA, the PCR examination has become a routine procedure out of all the available direct pathogen detection techniques such as immuno-fluorescent methods based on a more subjective evaluation or the longer process of culture. Reliable laboratory results can only be expected if samples have been collected and sent to the laboratory before any antibiotic therapy, as it may interfere with the successful detection of chlamydial DNA and may cause false-negative PCR results.
The increased level of specific IgM concomiting the invasive RTI can be detected using serological methods, which may be a useful diagnostic tool even in more chronic, pretreated cases, when the sensitivity of PCR has already decreased due to the absence of chlamydial DNA.

1.2.2. Infections caused by L serotypes: lymphogranuloma venereum (LGV)

LGV is a sexually transmitted disease caused by *Chlamydia trachomatis* serotypes L1-3. The clinical picture of LGV is characterised by various, often non-specific anogenital symptoms. A primary anogenital ulcer or erosion develops on the initial site of entry of the invasive LGV strains causing urethral/rectal discharge and a secondary purulent inguinal lymphadenopathy spreading via lymphatics. The classic form of the untreated disease typically follows a three-stage course.
LGV is endemic in tropical, subtropical regions, and only rare, imported cases had been diagnosed in Europe before 2003. Recent outbreaks of LGV proctitis among homo-/bisexual men in Rotterdam were, however, reported in 2003-2004 and since then the epidemic LGV strains have spread all over the European countries threatening mostly the afore mentioned high-risk population [16,17]. Asymptomatic LGV carriers may also serve as an infectious source of the disease. Based on literature data as many as a quarter of rectal LGV carriers may remain asymptomatic after being infected, underlying that the contact tracing and screening of risk-groups are of great importance [16].

1.2.2.1. Diagnostic relevance of the systemic immune response in LGV patients

In contrast to the acute, non-complicated, uro-/anogenital and conjunctival *C. trachomatis* infections caused by D-K serotypes and normally confined to the mucosal epithelium, the invasive L serotypes (LGV strains) can cross the epithelium and elicit a strong humoral

immune response [18]. While the majority of the detectable chlamydial antibodies secreted in the genital tract belong to the IgG class and originate from the circulation, the rectal mucosa contains a higher proportion of IgA-producing lympho-epithelial structures (in Peyer's patches). The latter are activated during the LGV infection and the increased level of produced antibodies can be detected with serological assays [19,20].

Following an LGV infection the elevated anti-*C. trachomatis*-IgA and -IgG may persist for years, indicating recent or past infections. The detection of a high level of specific IgA may be an early serological sign of an LGV infection as well as a helpful tool to differentiate it from a chlamydial infection due to D-K serotypes. A strong IgA and IgG seropositivity combined with clinical signs, suggestive of LGV in high risk MSM groups, may help to recognize an actual infection and indicates the further testing of relevant anogenital samples. Serological assays may provide a presumptive diagnosis completing the first-line approaches focusing on the detection of LGV biovar specific DNA [3,20,21].

1.2.2.2. Possibilities of laboratory diagnostics in LGV patients

The definite diagnosis of LGV requires targeted molecular biological assays even for testing clinically suspicious cases of high risk sexual behaviour, as the identification of the supposed etiological agent at a biovar level is essential for it. Relevant samples as per the actual symptoms are needed for confirmation, which most often include ulcer material from primary, anogenital lesions or anorectal specimens or inguinal bubo-aspirates.

Etiological identification usually follows a two-step procedure: after screening the clinical samples for the presence of *C. trachomatis* DNA by a conventional PCR, positive DNA-samples can be further evaluated by an LGV-specific real-time PCR, while the actual genotype can be determined by sequencing [17].

Besides direct detection techniques, serum samples of LGV patients are also suitable for the testing of specific IgA and IgG applying various serological assays (enzyme-linked immunosorbent assay [ELISA], micro-immunofluorescence [MIF] or immunoblot) since LGV, being an invasive type of chlamydial infection, is characterised by an intense, systemic immune response [21]. The detectable high levels of specific antibodies suggest a strong possibility of an LGV infection and suggest a repeated sampling and PCR test in case it has failed for any reason. The detection of humoral immune response, especially regarding the IgA of predictive value, may contribute to a presumptive diagnosis of LGV before the

identification of the actual genotype has occurred, in accordance with the recommended current LGV guideline of International Union against Sexually Transmittes Infections (IUSTI) [17].

1.3. *Treponema pallidum* subspecies *pallidum* infection: syphilis

Syphilis is the third most common bacterial STI in the world exceeded only by *C. trachomatis* infections and gonorrhoea [1]. Trends since 2011 reveal that syphilis rates have been increasing, particularly among men, mainly due to an increase among MSM. As the 2015 report of European Centre for Disease Prevention and Control (ECDC) shows, as many as 62% of the cases belonged to the MSM group, while only 9% of the cases belonged to females. Infected MSM and heterosexual men represented 8,5 times more cases than women alone [22].

Syphilis is a chronic invasive infection caused by *T. pallidum* subsp. *pallidum (T. pallidum).* The untreated disease is characterised by several stages and undulant clinical symptoms, starting with a local, painless ulcer on the site of entry, followed by generalised skin eruptions and mucosal signs, resulting in late organ damages. Neurological involvement (neurosyphilis) may develop at any stage of the infection [23].

The efficient recognition of gestational syphilis is affected by several diagnostic pitfalls. Spontaneously healing, primary chancres in females are frequently present in hidden anatomical sites (such as the vulva, cervix or pharynx); while due to the variable, often atypical presentations of secondary syphilitic mucosal or skin lesions, a misdiagnosis with allergic or toxic diseases is a common occurrence; and during the latent early or late phases of the infection the absence of symptoms may fail to alert the clinicians [24].

In the latent phases syphilis seropositivity may be revealed almost exclusively by screening tests or by contact tracing, which makes the routine prenatal screening a very effective means not only of the prevention of congenital syphilis but also of the recognition of the maternal infection [25].

A thorough venereological examination and exploring the anamnestic data about a risk of a potential exposure may call for targeted laboratory tests, which can focus either on the direct pathogen detection or serological tests or include both of these depending on the suspected stage of the infection [25]. Despite the massive immune response developing against *T. pallidum*, the etiologic agent of syphilis, no protective immunity exists in humans, who can be

infected several times during their lifetime [26]. During the course of infection dynamic changes of antibody levels can be observed in the majority of patients and the follow-up of their semiquantitative values (titres) helps to judge the effectivity of the treatment or the other way round, even the possibility of another reinfection. Consequently, the laboratory diagnostic methods combining various serological assays play a crucial role in establishing the actual stage of syphilis in patients [4,25].

1.3.1. Relevance and prevention of vertical transmission of *T. pallidum*

Based on estimated WHO data over 900 000 pregnant women were infected globally with syphilis in 2012 resulting in approximately 350 000 adverse birth outcomes due to transplacental transmission, threatening the health of the mothers themselves in untreated cases [27]. Congenital syphilis is the most severe manifestation of vertically transmitted bacterial STIs, which may cause systemic organ damages of the fetus leading to serious, irreversible malformations or even stillbirth. Women suffering from syphilis may infect their fetuses any time during their pregnancy. The majority of fetal infections are of haematogenic origin, while a primary chancre developing in the birth canal may serve as a source of a direct contact transmission extremely rarely [28].

The possibility of a vertical transmission is 70-100% in the primary/secondary stage of gestational syphilis; it decreases to 40% in the early latent phase, followed by around 10% in late latent phase, but the risk should never be discounted as negligible [29]. The distribution of the potential fetal and neonatal consequences depends on the actual level of maternal infectivity. Untreated primary and secondary syphilis result in a 25% risk of stillbirth, a 14% risk of neonatal death, a 41% risk of giving birth to a live but infected infant and only every fifth pregnancy in this patient group ends up giving birth to a healthy, uninfected baby [28,30]. Congenital syphilis could be theoretically fully prevented with a comprehensive gestational screening and by an adequate treatment of seropositive women. Consequently the incidence of this condition can be regarded as a negative indicator of any prenatal health care system, while it will be always related to the actual prevalence of infectious syphilis among patients of a reproductive age [30].

Pregnant women who do not attend routine prenatal care or being infected but left undiagnosed after having been screened early in their pregnancy pose the greatest risk to their fetuses in terms of congenital syphilis.

1.3.2. Possibilities of laboratory diagnostics in gestational syphilis

The current prenatal syphilis screening is regulated by a recommendation in Hungary, and should be performed during the first prenatal visit [31], however, we still have no national reported data about the seropositivity rate among pregnant women in Hungary. In the absence of these data we cannot assess either the risk of a gestational syphilis or that of a consequent congenital infection.

The laboratory investigation of syphilis combines the direct pathogen detection techniques (PCR, dark-field microscopy) suitable for diagnosing the infectious phases and the various serological tests aiming the detection of the humoral immune response (Rapid Plasma Reagin [RPR], Venereal Disease Research Laboratory [VDRL], Treponema Pallidum Haemagglutination Assay [TPHA], ELISA, immunoblot etc.) The latter assays can be effectively performed a few weeks after exposure and they optimally include both aspecific tests (for reagin-type antibodies) as well as specific tests (for anti-treponemal immunoglobulins). **(Figure 2.)**

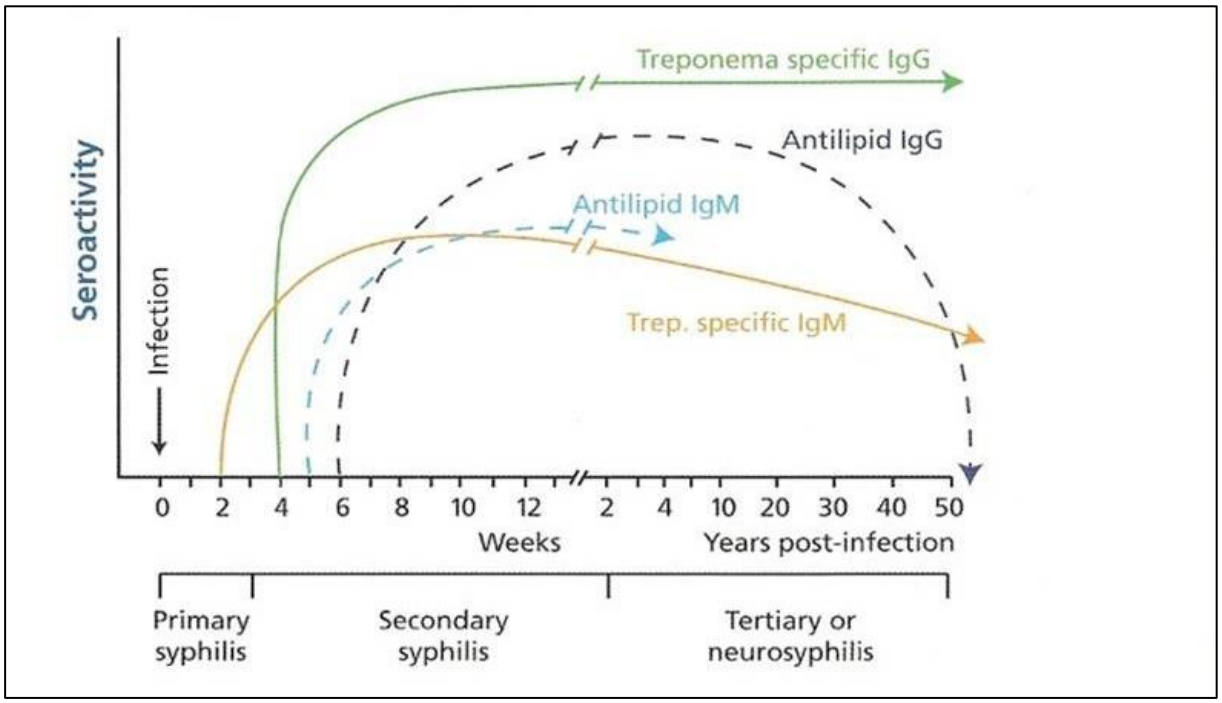

Figure 2. Dynamic changes of reagin-type (antilipid)- and treponemal antibodies belonging to classes IgM and IgG during the course of untreated syphilis [32]

Specific IgG is the serological marker of syphilis seroprevalence, i. e. the persistent clue of a past infection, that may be detectable even decades long; while the actual phase, i.e. the level of infectivity can be concluded from the level of the reagin-type, aspecific antibodies [4,14, 25].

Apart from the early, seronegative phase of the infection serological assays make up the „gold standard" laboratory diagnostic tool of syphilis, which are appropriate for screening and confirming gestational syphilis as well.

In the absence of an optimal and reliable method representing all the previous advantages of the combined testing in itself, one has several options to choose a diagnostical assay, depending on the supposed level of infectivity of the target groups, the possibilities of automatization of the tests and the estimated costs thereof.

Based on international guidelines low-risk populations, such as pregnant women can be effectively screened using the following algorithms:

- with treponemal tests performed in itself (mainly by ELISA);
- with combined treponemal and non-treponemal, (ideally quantitative) tests (such as a TPHA and titrated RPR test performed parallel) [4,14].

The sole application of non-treponemal tests (RPR, VDRL) is still practised in some countries, however this algorithm has several disadvantages, as only active, infectious syphilis can be detected by that way, while very early syphilis may be often missed. The reactivity of these tests indicates the infectivity of the patients and become usually negative after a past infection. Moreover, there is a common phenomenon of a „biological false positive" reaction (especially during pregnancy), which is characterised by the increased level of reagin-type antibodies. This false-positive reaction does not, however, point to an acute syphilis, which can be excluded with specific confirming assays and the repeated testing of paired sera [4].

At the same time, the combined or stepwise use of non-treponemal tests makes up an essential part of syphilis serology, since performed semiquantitatively they usually correlate with the disease activity, and their results (titres) can be used to monitor the disease-activity and the efficacy of treatment, as well as the chance of a potential reinfection. The follow-up of the reagin titres is crucial for to establish the actual phase of the disease [33].

One must note, however, that these follow-up procedures should preferably be always performed in the same laboratory, with the same tests as they need a manual and subjective

evaluation. Semiquantitative results of different tests done by different laboratories are not suitable for comparison [4].

Treponemal tests are suitable for the total (TPHA, ELISA) or separated (ELISA, immunoblot) detection of specific IgG and IgM antibodies but they are not helpful in assessing the disease activity and following treatment outcome. These tests are optimal for an automated large-scale screening of asymptomatic populations such as pregnant women of usually low seroprevalence. Although the specific IgM is the earliest serological sign of an infection and it enables the diagnosis of syphilis in its initial phase, its level does not necessarily become negative after an acute infection, unlike reagin titres. Specific IgG may remain positive for life in most patients, identifying persons with a previous successful treatment of syphilis as well as those with untreated syphilis [4,34]. (**Figure 2.**)

Independently of the chosen screening alternative all their indeterminate/reactive results should be confirmed with further assays based on another principle of antibody-detection, contributing to the exclusion or the confirmation of the infection by *T. pallidum*.

This protocol should be definitely followed when testing pregnant women and if necessary (e.g. in the case of indeterminate results; supposing an early, seronegative phase of an acute infection; in that of a differential diagnosis of a biological aspecific reaction etc.), a close monitoring and the testing of paired sera are needed. It should be emphasized that all the laboratory results targeting the diagnosis of syphilis (except for the positive *T. pallidum* PCR results of definite value) should be assessed together with the anamnestic data and the actual clinical signs and symptoms [24,35].

2. AIMS OF THE STUDIES

Our aims were the following:

2.1. Determination of *C. trachomatis* seroprevalence among neonates suffering from RTI

a) to determine the *C. trachomatis* seroprevalence among symptomatic infants suffering from RTI in Hungary, compared to data published in the literature;

b) to describe the epidemiological features (distribution of age, gender and the need for hospitalisation) of the seropositive patient group compared to patients suffering from ON;

c) to compare the actual chlamydial serostatus of the study group with their pertussis serostatus based on the laboratory database.

2.2. Detection of *C. trachomatis* specific immune response among LGV patients

a) to test the serum samples of LGV patients in Hungary with ELISA for the presence of anti-*C. trachomatis* IgA and IgG; as well as to evaluate the reactivity pattern of specific IgA and IgG by immunoblot technique (unprecedented worldwide, to the best of our knowledge);

b) to compare the levels of specific IgA and IgG of LGV patients with the anti-*C. trachomatis* IgA and IgG levels of a non-LGV patient group; as well as to compare the IgG immunoblot reactivity pattern of both groups. The non-LGV patients were represented by specific IgG seropositive, infertile women of similar age, infected by D-K serotypes.

c) to summarize the practical possibilities of applying the *C. trachomatis* immunoblot technique in the laboratory diagnosis of LGV.

2.3. Determination of *T. pallidum* seroprevalence among pregnant women

a) to determine the syphilis seroprevalence in a large prescreened group of pregnant women in Hungary during the period 2013-2016, verified for syphilis in our laboratory; as well as to compare these results with other seroprevalence data reported from other countries;

b) to determine the semiquantitative RPR titres among seropositive women and to assess the actual level of infectivity and the risk of a consequent transmission to their fetuses;

c) to analyse the age-distribution among seropositive women and to define the most threatened maternal age-group regarding getting infected by syphilis;

d) to analyse the given data of gestational age at screening among seropositive pregnant women, compared to the actual recommendation for prenatal screening;

e) to define the rate of women tested by comprehensive screening versus individual venereological investigation due to contact tracing; and to evaluate the efficacy of prenatal screening in revealing gestational syphilis.

3. MATERIALS AND METHODS

3.1 Determination of *C. trachomatis* seroprevalence among neonates suffering from RTI

3.1.1. Study group, clinical samples

During the period between January 2008 and December 2016 serum samples were collected from 262 neonates with clinical symptoms of RTI. The age of the enrolled subjects varied between 1 and 20 weeks. Age, sex and hospital admission (if any) were recorded. The blood samples were collected by neonatologists after diagnosing RTI on clinical (tachypnoea/persistent cough) and/or radiological signs (hyperinflation, diffuse pulmonary infiltrates). Forty-one percent of the patients (N=107) were treated in medical institutes in Budapest, while 59% of them (N=155) were referred from the countryside. Serum samples from infants with RTI were stored at -20 °C until further processing. Due to clinically similar symptoms the testing of all neonates in the study group was also indicated for pertussis.

3.1.2. Detection of *C. trachomatis* specific IgM

Due to previous recommendations a MIF test (Focus, Cypress, USA) was chosen as the gold standard method from all the available serological assays, used in accordance with the manufacturer's instructions to detect the *C. trachomatis*-specific IgM in these serum samples [36].

The assay was performed from 10 µl serum diluted with 150 µl Pretreatment diluent, and phosphate buffered saline (PBS) was used to determine the endpoint titres. Purified elementary bodies of 8 serotypes (D-K) of *C. trachomatis* served as chlamydial antigens. In positive cases the fluorescein-labelled antihuman IgM could react with the antigen-human IgM complexes. The cut-off was defined at a serum dilution rate of 1:32 considered to be diagnostic for the infection [18]. Samples of seropositive patients showed an intense, bright-green fluorescence under UV-microscope due to the binding of fluoresceine-labelled antihuman-IgM to the chlamydial antigen-human IgM complexes.

After the evaluation process, (strong) reactive (dilution titre ≥32), weak reactive (reactivity with dilution titre <32); and negative results were obtained. Based on these results, the patients were divided into 2 main groups, regarded seropositive exclusively at a dilution titre of 32 or more; while all the other (non-reactive, indeterminate or weak reactive) cases were interpreted seronegative. Even the latter ones were excluded from the further analysing

process as the real reason for a weak reactivity could not be reliably established, whether caused by a possible cross-reaction or a low level of true reactivity, so the chlamydial etiology could not be proven for them. All the serum samples were simultaneously tested with a *Bordetella pertussis* Indirect Immuno-Fluorescence Test (IIFT) (Euroimmun, Lübeck, Germany) for the presence of a specific anti-pertussis IgM (by Mrs. Ildikó Paluska, 2nd Dept. of Bacteriology, OEK) and all these results were registered in the laboratory database.

3.1.3. Statistical analysis

A Pearson's chi-squared test (χ^2) was used to examine the significance of the relationship between the respiratory tract infection by *C. trachomatis* and the gender of the neonates.

3.2 Detection of *C. trachomatis* specific immune response among verified LGV patients

3.2.1. Study group, clinical samples

Our study group consisted of verified LGV patients, whose anogenital samples had been found positive for *C. trachomaties* L1-3 by LGV real-time PCR and by genotyping. We have verified altogether 53 LGV cases between September 2012 and December 2017 in Hungary, of whom we had received 36 serum samples. These serum samples were stored at -20 °C until further processing. The age of the enrolled subjects (N=36) with available serum samples varied between 20 and 59 years. The median age in this group was 35.

3.2.2. Detection of *C. trachomatis* specific IgA and IgG by ELISA and by immunoblot tests

The serological tests were performed in two steps. First, all the serum samples (N=36) of LGV patients were tested for the presence of specific antibodies by a commercial *C. trachomatis* IgA and IgG ELISA (NovaTec, Dietzenbach, Germany), following the manufacturers' instructions. A further investigation was done with a specific IgA and IgG immunoblot assay (GenID Gmbh, Strassberg, Germany). The latter test is recommended for the laboratory diagnostical process of the ascending, chronic *C. trachomatis* D-K infections, which enables the detection of reactivity against the following antigens:

- some genus-specific antigens, such as chlamydial lipopolysaccharide (LPS) and heat shock-protein (HSP)60 (one of the main serological markers of tubal infertility); and

- some species-specific antigens: 40 kDa MOMP1, 29 kDa, 45 kDa and 80 kDa antigens [37,38].

The reactivity pattern of each immunoblot strip was compared to the pattern of the control strips provided with each kit, and was interpreted by a subjective evaluation, marked by: (+) when found weak reactivity; + when found equally reactive; ++ or +++ when strong or highly strong reactivity. (The latter designation was used when a confluent dark pattern was observed.) The results of the IgA and IgG ELISA, together with those of the IgG immunoblot of LGV patients were compared to the results of a group of infertile women (N=36) having been previously infected with an ascending type of *C. trachomatis* D-K, and tested with the same serological assays.

3.2.3. Statistical analysis

A two-sample Mann-Whitney U-test was used to compare the ELISA IgA and IgG COI (cut-off-index) values of the serum samples in the LGV group (N=36) versus the infertile women (N=36)

3.3 Determination of *T. pallidum* seroprevalence among pregnant women

3.3.1. Study group, clinical samples

Large scale serum samples of 49 965 pregnant women were tested by an automated ELISA (Trepanostika TP, MicroELISA BioMérieux, France) for the presence of specific *T. pallidum* IgG/IgM antibodies (by Erzsébet Barcsay MD, OEK, Dept. of Virology) between 2013-2016. All the samples showing reactivity (N=527) were submitted for further verification of *T. pallidum* infection to our laboratory. The serum samples were stored at -20 °C until further processing.

3.3.2. Serological methods applied for *T. pallidum* diagnostics

Confirmation process included a stepwise application of combined serological assays of different principle (**Figure 3**.) First, the combination of a titrated Rapid Plasma Reagin (RPR) test (Omega Diagnostics, Alva, Scotland) with a two-fold dilution method and a qualitative Treponema Pallidum Haemagglutination Assay (TPHA) (Trinity BioTech, Bray, Ireland) in a dilution of 1:80 was applied. When both of these tests were found negative, the samples were judged negative and no further tests were performed as they had no serological signs of a past

or an acute syphilis. When any of these tests were found indeterminate or positive, anti-*Treponema pallidum* IgG and IgM ELISA (Euroimmun, Lübeck, Germany) tests were performed. All women interpreted as syphilis seropositive had specific anti-Treponemal IgG detected by ELISA. The end-titres were also evaluated by a two-fold dilution method of the sera showing some RPR activity. A dilution of 1:8 was chosen as the diagnostic cut-off, as RPR>8 titres are highly suggestive of an acute/recent infection [14,32,39]. (**Figure 3.)** Based on diluted RPR results (negative; 1≤ 8; or >8) the seropositive patients were divided into 3 groups.

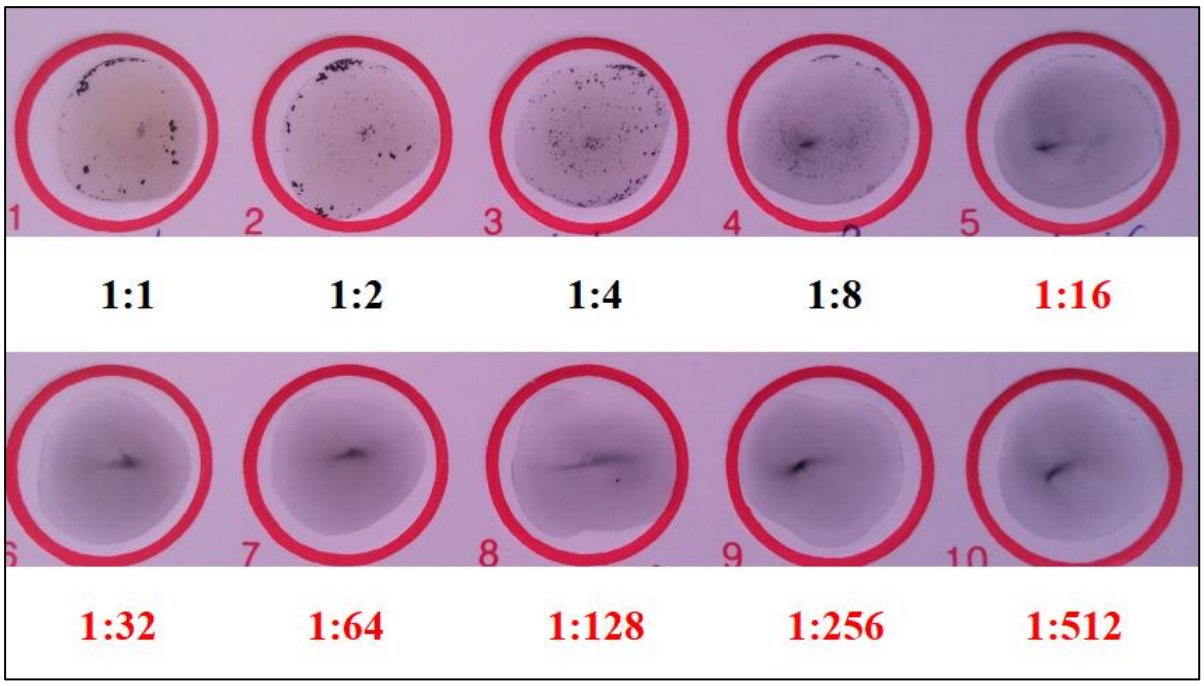

Figure 3. Dilution row of an RPR-positive serum sample (own picture)
(titres higher than the diagnostic cut-off of 8 marked by red numbers)

During the anonymous analyses of personal data reported by the clinicians only the age and the estimated gestational age at sampling were taken into account. Concerning the indication for syphilis testing the seropositive patients were revealed either as a result of the recommended prenatal screening or due to an individual, random investigation during a targeted contact tracing. There was no overlapping within these two groups of indications, as the latter group was referred for syphilis screening by dermato-venereologists before a general screening could have been performed, or instead thereof.

Based on personal consultation data, all the results in our study were the first antenatal testing data, reflecting the serostatus of the first screening in every pregnancy. No results of any later

follow-up or repeated tests were included in this study when retesting occurred among the analysed seropositive group. The algorithm of the stepwise applied and combined syphilis tests, as well as the interpretation of their results are detailed in **Figure 4.**

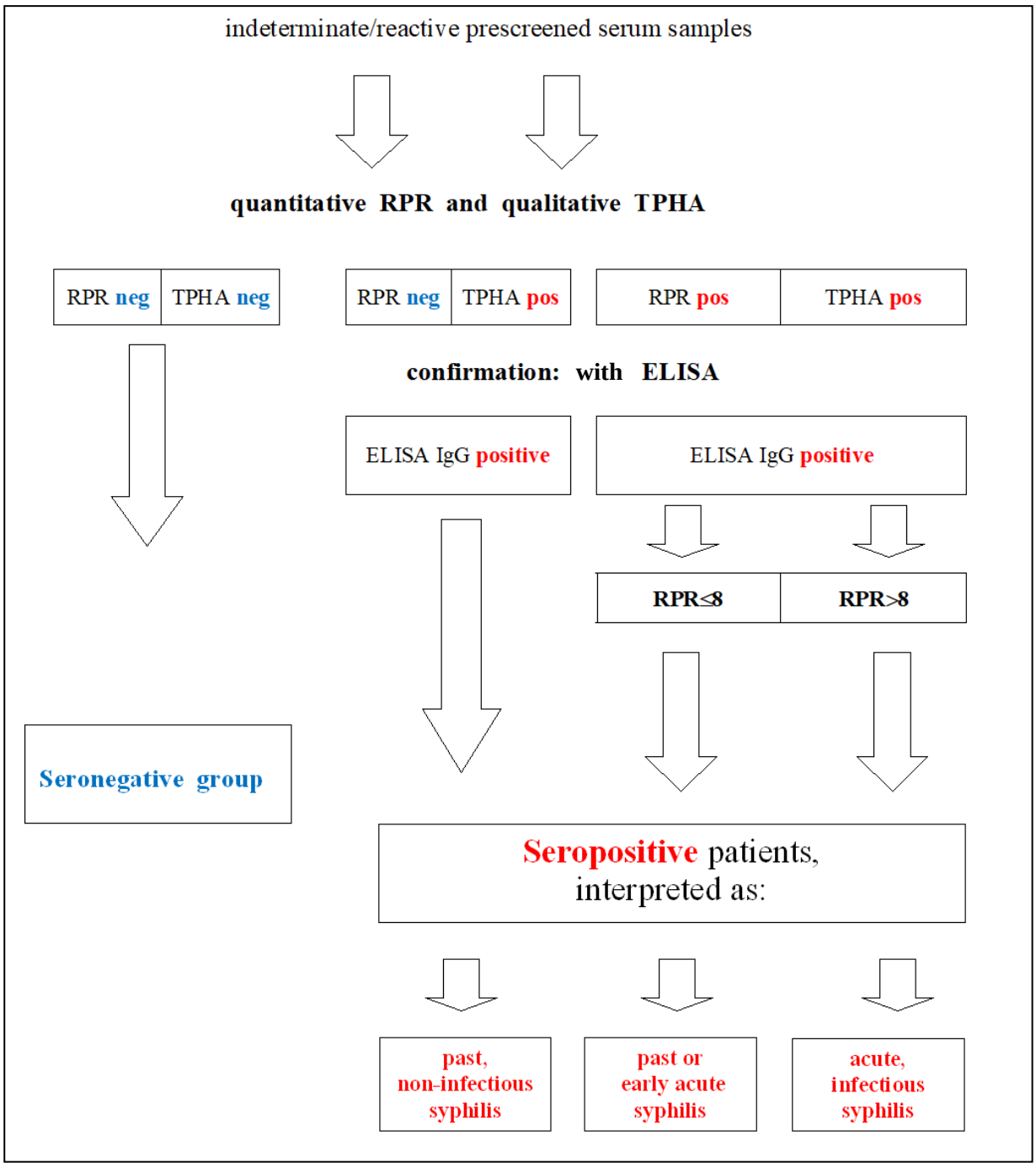

Figure 4. Algorithm of the laboratory diagnosis of gestational syphilis performed with *T. pallidum* serological tests (Abbreviations: pos- positive, neg- negative)

4. RESULTS

4.1 Determination of *C. trachomatis* seroprevalence among neonates suffering from RTI

4.1.1. Serological results, seroprevalence

The male to female ratio of symptomatic infants with RTI was 149 male vs 113 female infants (ratio 1.3). Two hundred and thirteen out of 262 affected infants (81.3 %) were hospitalized (126 male versus 87 female, ratio 1.4). Data are shown in **Table 1.**

Seronegative infants represented 65.3 % (171/262) of the group, a weak reactivity was detected in 15.6 % (41/262), while 19.1 % (50/262) of patients were found unequivocally seropositive when tested in the chosen dilution (diagnostic cut-off of 1:32).

The distribution of data of the seropositive group (N=50) is highlighted in the row with bold borders in **Table 1.** According to these results seroprevalence was found 19% (50/262) in this study group.

***C. trachomatis* IgM**	**Hospitalized patients (N=213)**		**Outpatients (N=49)**		**All**
	males	**females**	**males**	**females**	
seronegative	101	72	16	23	212 (81%)
seropositive	**25**	**15**	**7**	**3**	**50 (19%)**
All	**126** (48%)	**87** (33%)	**23** (9%)	**26** (10%)	**262**

Table 1. Distribution of gender, need for hospitalization and of the serostatus of *C. trachomatis* IgM among neonates suffering from RTI (N=262)

4.1.2. Characterisation of seropositive patients

The data of specific IgM-positive neonates (N=50) were further analysed according to gender, postnatal age as well as the need for hospital care, if any. The age of the seropositive patients ranged between 3-20 weeks, the median age being 9 weeks. The gender distribution showed an even stronger male dominance of 32 males vs 18 females (ratio 1.8) than the gender distribution of the whole study group (ratio 1.3).

Eighty percent of seropositive babies needed hospital care (40/50), again with a male dominance (25 males vs 15 females, ratio 1.7). The age- and sex-distribution, as well as the inpatient/outpatient rates are outlined in **Table 2.**

Presumably due to the low number of patients we could not confirm any significant relation between gender and infection (p=0.26).

The pertussis serostatus for the study group was also checked in the laboratory database but none of the serum samples were reported positive when tested by the anti-*Bordetella pertussis* IgM IIFT, i.e. *B. pertussis* as an etiological agent could be excluded from a differential diagnostical point of view.

Postnatal age (weeks)	**Number of inpatients (N=40)**		**Number of outpatients (N=10)**		**All**
	males	**females**	**males**	**females**	
3-4	6	3	0	1	10
5-6	5	4	1	0	10
7-10	5	4	3	0	12
11-14	5	1	1	2	9
15-20	4	3	2	0	9
All	**25**	**15**	**7**	**3**	**50**

Table 2. Distribution of age, gender and need for hospitalization among *C. trachomatis* IgM-positive neonates (N=50)

4.2. Detection of *C. trachomatis* specific immune response among verified LGV patients

The age spectrum of the patients involved in the serological investigation (N=36) showed an equal distribution throughout the age groups (median age: 35): 12 cases belonged to 20-30 year old group; 13 cases belonged to the 30-40 year old group, while 11 cases to the age group older than 40.

The distribution of the main clinical manifestations of the LGV infection were as follows: proctitis as a leading sign in 27 patients (75%); inguinal lymphadenopathy in 7 patients (19.4%) and urethritis in 2 patients (5.6%).

4.2.1. ELISA results

All the tested serum samples (N=36) proved strongly positive for both *C. trachomatis* IgA and IgG. The ELISA results are summarized in **Table 3.**, calculated in cut-off index (COI) to facilitate an easier comparison. The following formula: $COI = \frac{OD_{sample}}{OD_{cutoff}}$ was used to determine the ratio of the optical density of the tested serum sample and of the mean optical density value of the cut-off-calibrators (given in each kit).

The COI values of the IgA ELISA results fell in the range of 1.2 to 11 with a median COI of 3.5. The COI values of the IgG ELISA results fell in the range of 1.4 to 8.5 with a similarly high median COI of 3.3.

4.2.2. Immunoblot results

As expected, due to the high IgA and IgG ELISA COI values all the tested serum samples (N=36) yielded a robust, or even confluent reactivity pattern both on IgA and IgG immunoblot strips. Prominent reactive bands were observed to the 40 kDa major outer membrane protein 1 (MOMP1), to the 45 kDa protein, to the 80 kDa protein, moreover to the heat shock protein 60 kDa (hsp60) with a slightly less reactivity in the region of the 29 kDa protein. **(Figure 5.)**

We have summarized the COI values of IgA and IgG ELISA tests; the evaluated reactivity to certain chlamydial antigens listed above by IgA and IgG immunoblot; as well as the patients' age and their main clinical symptoms (anal, inguinal, urethral) in **Table 3**.

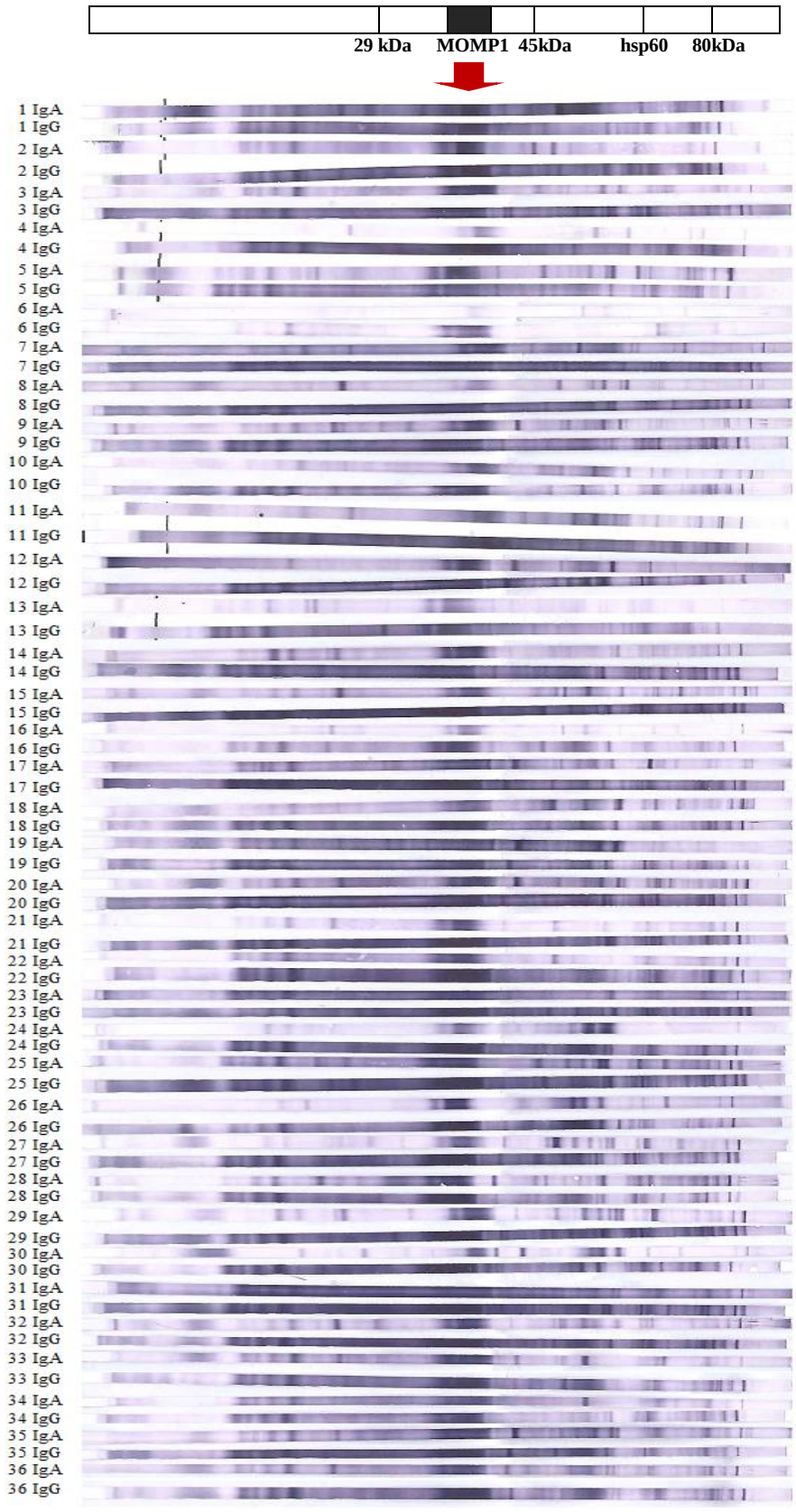

Figure 5. *C. trachomatis* IgA és IgG immunoblot reactivity patterns of LGV patients (N=36) (MOMP1 antigen marked with a red arrow)

Nr.	main clinical sign	age	CT ELISA		CT-immunoblot reactivity									
			COI		29 kD		MOMP1		45 kD		HSP60		80 kD	
			IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
1	I	35	7.6	4.1	(+)	++	+++	+++	+	+	(+)	+	+	++
2	I	28	2.6	2.5	(+)	(+)	+++	+++	(+)	(+)	(+)	+	(+)	++
3	A	41	2.9	3.9	(+)	+	+++	++	(+)	++	+	+	(+)	+
4	U	29	1.8	1.9	-	+	+	+++	(+)	+	(+)	+	(+)	+
5	I	20	1.7	3.8	-	(+)	+++	+++	+	+	++	+	+	+
6	A	31	1.7	2.3	(+)	(+)	+	++	(+)	-	(+)	(+)	(+)	(+)
7	A	45	9.5	8.5	(+)	+	+++	++	+	++	+	+	+	++
8	A	41	4.6	6.2	(+)	+	++	++	(+)	++	+	++	(+)	+
9	A	41	6.0	6.9	+	+	+++	++	++	+	++	+	+	++
10	A	24	3.9	5.6	(+)	(+)	+++	+++	(+)	-	+	+	+	+
11	A	28	5.3	3.9	(+)	+	++	+++	+	+	+	(+)	+	+
12	A	47	11.0	6.2	(+)	+	+++	+++	++	++	++	++	(+)	+
13	I	36	2.9	2.2	(+)	+	++	+++	(+)	(+)	+	(+)	(+)	+
14	A	35	6.1	7.9	(+)	+	+++	++	(+)	++	+	++	+	+
15	A	41	5.3	4.3	-	+	+++	+++	(+)	+	+	++	+	+
16	U	44	2.0	1.8	-	(+)	++	+++	(+)	(+)	(+)	(+)	(+)	(+)
17	I	27	6.0	4.5	+	+	+++	+++	++	+	+	(+)	(+)	+
18	A	42	2.6	3.2	(+)	+	++	+++	(+)	(+)	++	++	(+)	+
19	A	25	6.1	3.7	+	(+)	+++	+++	++	++	++	+	(+)	+
20	A	24	2.3	4.3	(+)	(+)	+++	+++	++	(+)	+	++	+	++
21	A	45	2.3	2.7	(+)	(+)	+++	+++	(+)	(+)	(+)	++	+	++
22	A	35	2.4	1.4	(+)	(+)	+++	+++	(+)	(+)	(+)	(+)	+	++
23	A	26	4.2	3.4	(+)	(+)	++	+++	(+)	(+)	++	+	+	+
24	I	39	5.5	3.7	-	(+)	+++	+++	(+)	(+)	++	++	(+)	++
25	A	39	4.3	4.0	(+)	(+)	+++	+++	(+)	(+)	++	++	+	+
26	A	31	2.7	2.8	-	-	+++	+++	(+)	(+)	++	++	(+)	+
27	A	59	5.2	4.6	(+)	(+)	+++	+++	+	+	++	+	(+)	+
28	A	33	5.4	1.6	+	+	+++	+++	(+)	+	++	(+)	+	+
29	A	28	3.2	2.8	(+)	+	+++	+++	(+)	(+)	+	++	(+)	+
30	A	28	1.5	3.1	(+)	+	++	++	+	(+)	++	++	(+)	++
31	I	35	4.6	2.5	(+)	+	+++	+++	+	(+)	++	++	++	++
32	A	33	3.1	2.8	(+)	+	+++	+++	(+)	(+)	+	+	+	++
33	A	41	2.5	2.8	+	(+)	+++	+++	+	(+)	-	(+)	(+)	+
34	A	35	1.2	2.0	(+)	(+)	++	+++	-	(+)	-	(+)	+	+
35	A	36	3.8	2.7	(+)	+	+++	+++	++	++	++	++	+	(+)
36	A	24	2.0	2.5	(+)	+	+++	+++	++	+	(+)	(+)	+	++

Table 3. *C. trachomatis* IgA and IgG ELISA COI values and interpreted immunoblot results of verified LGV patients (N=36) characterised by age and main clinical signs

Abbreviations: CT: *Chlamydia trachomatis*; A: anal; I: inguinal, U: urethral;
(+) : weak positive; +: positive; ++: strong positive; +++: highly strong positive

4.2.3. Comparison of serological results of LGV- and non-LGV patients

The IgA and IgG ELISA COI values, as well as the detailed reactivity of IgG immunoblot strips of *C. trachomatis* D-K infected infertile women (N=36) are summarized in **Table 4.**

Nr.	age	ELISA IgA		ELISA IgG		IgG immunoblot reactivity				
		COI	result	COI	result	29 kD	MOMP1	45 kD	HSP60	80 kD
INF01	36	1.6	**positive**	1.4	**positive**	+	++	+	+	+
INF02	36	0.9	indeterminate	1.8	**positive**	(+)	++	+	+	+
INF03	36	0.8	negative	2.2	**positive**	+	++	(+)	+	+
INF04	37	0.6	negative	1.3	**positive**	(+)	+	+	+	(+)
INF05	33	0.6	negative	1.1	indeterminate	+	++	+	+	+
INF06	33	0.9	indeterminate	1.4	**positive**	+	++	+	+	+
INF07	35	0.7	negative	1.3	**positive**	(+)	++	+	+	+
INF08	42	1.9	**positive**	2.1	**positive**	+	++	(+)	+	+
INF09	45	1.0	indeterminate	1.1	indeterminate	(+)	++	(+)	+	(+)
INF10	49	2.0	**positive**	2.0	**positive**	(+)	++	(+)	+	+
INF11	38	0.8	negative	1.5	**positive**	(+)	++	+	+	(+)
INF12	43	2.3	**positive**	1.8	**positive**	+	++	+	+	(+)
INF13	23	1.6	**positive**	1.2	**positive**	(+)	++	+	++	(+)
INF14	33	2.6	**positive**	1.4	**positive**	+	++	+	++	+
INF15	37	1.7	**positive**	2.5	**positive**	+	++	+	+	+
INF16	38	1.5	**positive**	1.2	**positive**	(+)	++	+	(+)	(+)
INF17	34	0.6	negative	1.5	**positive**	(+)	++	(+)	+	(+)
INF18	40	1.4	**positive**	1.5	**positive**	(+)	++	(+)	+	+
INF19	26	0.7	negative	1.1	indeterminate	(+)	++	+	+	(+)
INF20	42	0.6	negative	1.2	**positive**	(+)	+	(+)	+	+
INF21	32	1.3	**positive**	0.9	indeterminate	+	++	(+)	+	(+)
INF22	36	0.6	negative	0.9	indeterminate	+	++	(+)	+	(+)
INF23	40	0.5	negative	1.1	indeterminate	(+)	++	(+)	+	(+)
INF24	38	0.9	indeterminate	0.9	indeterminate	(+)	++	+	(+)	+
INF25	33	1.7	**positive**	1.1	indeterminate	+	++	(+)	+	+
INF26	45	2.3	**positive**	2.0	**positive**	(+)	++	+	+	+
INF27	22	1.7	**positive**	1.7	**positive**	(+)	++	+	(+)	+
INF28	39	1.3	**positive**	1.3	**positive**	(+)	++	(+)	(+)	+
INF29	27	2.1	**positive**	1.6	**positive**	(+)	++	+	+	(+)
INF30	42	1.3	**positive**	3.3	**positive**	+	++	++	++	+
INF31	39	2.2	**positive**	1.9	**positive**	(+)	++	(+)	++	+
INF32	30	0.3	negative	1.3	**positive**	(+)	++	(+)	+	+
INF33	38	1.0	indeterminate	0.9	indeterminate	(+)	++	(+)	+	+
INF34	27	0.4	negative	2.0	**positive**	(+)	++	(+)	+	(+)
INF35	34	1.4	**positive**	1.5	**positive**	(+)	+	(+)	+	(+)
INF36	23	1.2	**positive**	1.7	**positive**	(+)	++	+	(+)	+

Table 4. Interpreted *C. trachomatis* IgA and IgG ELISA COI values and interpreted IgG immunoblot results of infertile patients (N=36) characterised by age

This non-LGV study group (median age: 36) yielded indeterminate/ positive *C. trachomatis* IgA results in 66% of samples (median COI: 1.25; range: 0.3-2.6). An indeterminate *C. trachomatis* IgG value was detected in 25% of the samples, while 75% of them proved positive for *C. trachomatis* IgG (median COI: 1.4; range: 0.9-3.3). The comparison of the distribution of ELISA COI values of the two patient groups is shown in the **Figure 6.**

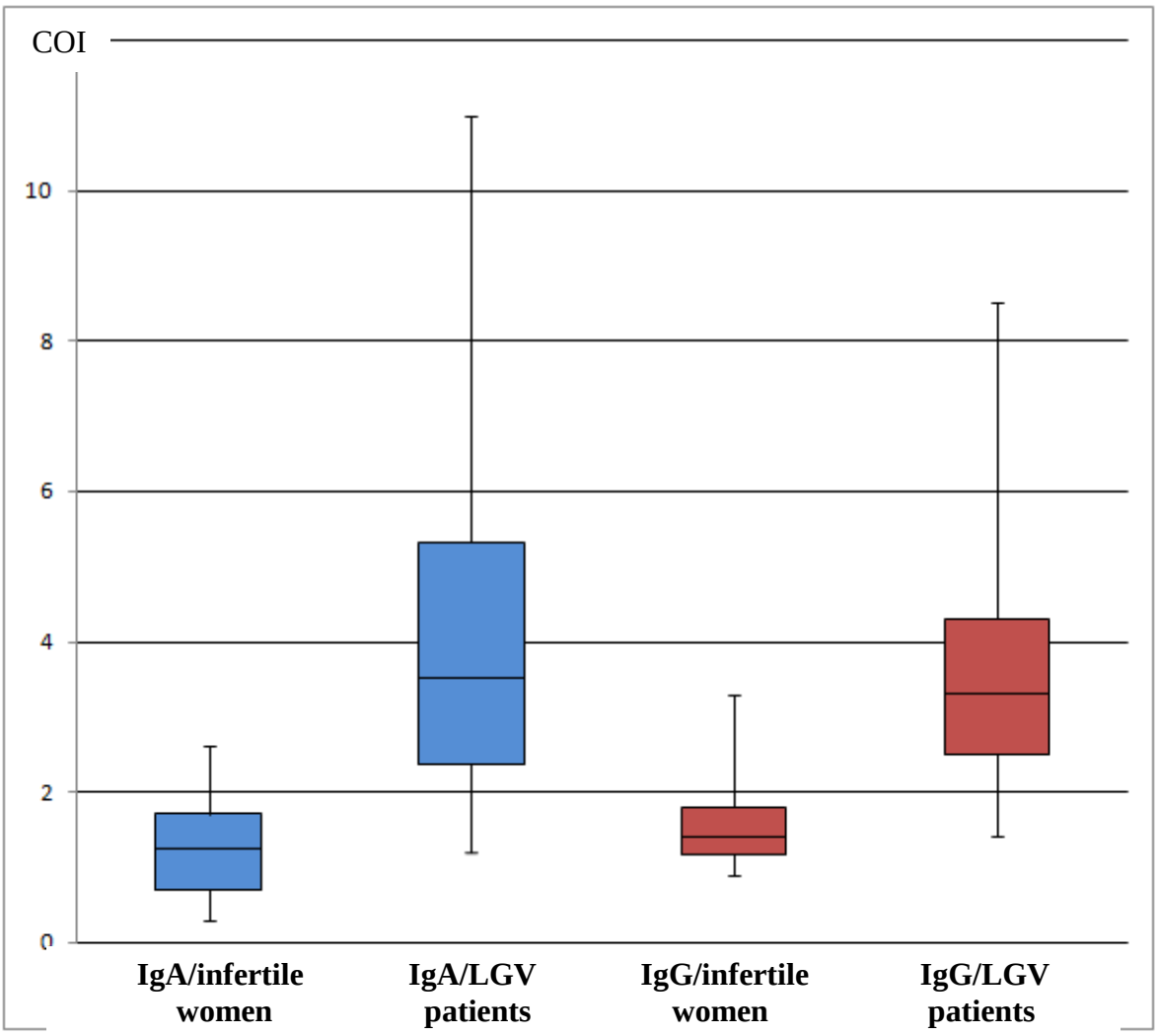

Figure 6. Distribution of *C. trachomatis* ELISA IgA and IgG COI results of infertile women (N=36) versus LGV patients (N=36)

The median IgA COI of LGV patients was almost 3 times higher than that of infertile patients (3.5/1.25=2.8), while regarding the levels of median IgG COI this ratio was also more than twofold that of infertile patients (3.3/1.4=2.35). Due to the two-sample Mann-Whitney U-test used to compare the ELISA IgA and IgG COI values, both IgA (U-score=73, $p<0.001$), and IgG levels (U-score=106.5, $p<0.001$) were found significantly higher among LGV patients versus infertile women.

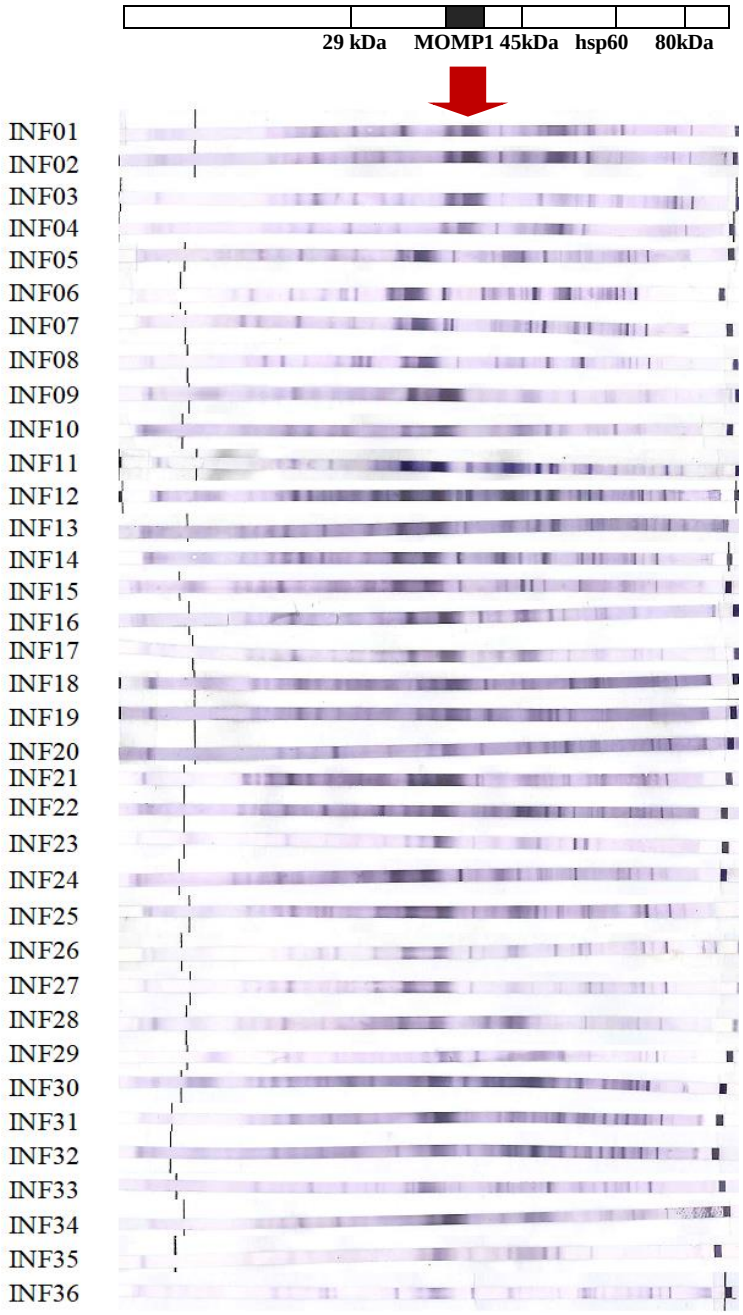

Figure 7. ***C. trachomatis*** **IgG immunoblot reactivity pattern of infertile women (N=36) (MOMP1 antigen marked by a red arrow)**

As all the samples of the infertile women were found positive for *C. trachomatis* IgG showing reactivity by immunoblot (**Figure 7.**) they became suitable for comparison with the immunoblot results of the LGV patients. Comparing the immunoblot patterns there were no differences on antigen levels between the positive IgG results of the LGV and that of the infertile patients, while the LGV patients' samples showed a much more robust intensity. This

was observed especially remarkably in the region of the MOMP1 antigen, supported by the detailed marks of our interpretation (**Table 3**. and **4**.)

4.3. Determination of *T. pallidum* seroprevalence among pregnant women

Altogether one hundred and forty eight women proved seropositive (specific IgG positive) tested by *Treponema pallidum* ELISA during this 4 year period (global seroprevalence 2.9‰). Seroprevalence was found 2‰ in 2013 (14/6800), 2.7‰ (32/11560) in 2014, 3.4 ‰ (49/14348) in 2015 and 3‰ (53/17257) in 2016, respectively.

The potential infectivity of the seropositive samples was assessed by the parallel detected RPR reactivity with a chosen cut-off of higher than a 1:8 dilution degree. Based on the observed RPR titres the seropositive pregnant women were divided into 3 groups (**Table 5.**):

1) RPR-negative cases indicative of a past infection were found in 53 women (36%);

2) weakly reactive RPR (titres≤1:8), referring to as either a past or an early acute infection, was observed in 55 women (37%). We could not conclude any more relevant information in the absence of any monitoring of paired sera of this group, which would have been useful to follow up the initial RPR titres and to establish a more reliable diagnosis.

3) strong RPR reactivity (titres>8), suggestive of a recent, infectious syphilis, was present in 40 (27%) of the 148 seropositive women. Half of the latter (20/40) belonged to the age group of 15-24 years (highlighted in grey in Table 5.), while we found 6 cases of 40 suffering from an active, infectious syphilis even in the age group of 35 years or above.

RPR titre \ Age	15-19	20-24	25-29	30-34	35≤	ALL
negative	4	10	7	12	20	53 (36%)
positive≤8	11	15	13	6	10	55 (37%)
positive>8	6	14	7	7	6	40 (27%)
ALL	**21**	**39**	**27**	**25**	**36**	**148**

Table 5. Distribution of RPR titres in the age groups of seropositive pregnant women (N=148)

The gestational age at sampling was reported in 123 of the 148 seropositives cases. Seropositivity was diagnosed in their second trimester in 56 of them (45.5%), and in the third trimester in 27 of them (22%). Of these 27 seropositive women 7 (26%) had serological evidence of a recent, infectious syphilis (**Table 6.**).

Gestational age / RPR titre	**I. trimester**	**II. trimester**	**III. trimester**	**All**
RPR negative	13	21	7	41 (33%)
RPR≤8	10	26	13	49 (40%)
RPR>8	17	9	7	33 (27%)
All	**40 (32.5%)**	**56 (45.5%)**	**27 (22%)**	**123**

Table 6. Distribution of RPR titres in the reported trimesters of seropositive pregnant women (N=123)

Of all the seropositive cases, 19 (13%) originated from a random venereological screening, while 129 (87%) were detected as a result of a routine prenatal screening (**Table 7**). Altogether 29 (72.5%) of all patients suffering from a presumably acute, infectious syphilis were diagnosed due to prenatal screening.

Indication for screening / RPR titres	Routine prenatal screening	Random venerological screening
RPR negative	50	3
RPR≤8	50	5
RPR>8	**29**	**11**
All: 148	129 (87%)	19 (13%)

Table 7. Distribution of RPR titres and indications for screening in seropositive pregnant women (N=148)

5. DISCUSSION

5.1. Determination of *C. trachomatis* seroprevalence among neonates suffering from RTI

RTI is one of the most common neonatal diseases that may be caused by a wide range of pathogens and via different transmission routes. The infected birth canal itself may play an important etiological role as a vertical transmission of frequently transmitted bacteria (e.g. *Streptococcus agalactiae*, *Enterobacteriaceae* spp, etc.) and *C. trachomatis* can occur [40]. As one of the major pathogens causing sexually transmitted infection, *C. trachomatis* is responsible for an estimated 146 million infections per year worldwide, particularly affecting young people aged 15-28 years [1,6]. The likelihood of women becoming pregnant is increased in this age group and those that do may become a maternal source of a potential *C. trachomatis* infection in newborns. The transmission rate from mother to child varies between 50-70%, and the most severe clinical manifestation, RTI, develops in 10-20% of the infected neonates [9,10]. The estimated prevalence rates of neonatal *C. trachomatis* infections range from 4-60 per 1000 live births in developed countries [9].

The “classic” type of chlamydial infantile pneumonia is usually a late onset disease, typically developing at 4-12 weeks of age, accompanied by a so-called “staccato” cough similar to pertussis [13]. Rhinorrhoea may be a characteristic prodromal sign, but fever is infrequent [41]. ON is present in about half of the cases, which is a valuable diagnostic clue, together with peripheral eosinophilia [15,36,42].

As the majority of clinical symptoms and radiology signs are nonspecific, the differential diagnosis should be extended to the most prevalent viruses (respiratory syncytial virus [RSV], adenovirus, cytomegalovirus etc.) and bacteria (*Streptococcus pneumoniae, Staphylococcus aureus,* etc.), which can also predispose individuals to late onset respiratory infections [43,44,45]. One should note that mixed infections with these respiratory pathogens may also occur and viral co-infection has been reported in a higher proportion of severe cases compared to cases of mild pneumonia [13,46].

As the clinical signs often resemble pertussis, this must also be considered, especially in unvaccinated young infants. Its real risk depends on the actual epidemiological situation, principally when similar cases are accumulating and/or no vaccination can be assumed among the contacts. In our series, we did not find any confirmed pertussis among the study patients.

The gold standard for the laboratory diagnosis of *C. trachomatis* RTI is the PCR examination of the nasopharyngeal aspirates (or invasively taken respiratory tract samples in ventilated patients) for the presence of the pathogen, but only if collected before starting an antibiotic therapy, to prevent false-negative PCR results [14]. In this study most of the infants had already received some antibiotic treatment, rendering the negative results from PCR testing unreliable. For that reason these types of clinical samples were not collected from the majority of our patients.

In the absence of relevant respiratory samples the specific IgM response can be detected with serological assays as a supportive test. As the maternal IgM does not pass across the placenta, the presence of *C. trachomatis* specific IgM antibody in newborns' blood samples is indicative of a systemic immune response of the neonate elicited by this invasive type of infection [3,15].

The seronegative or weak seropositive *C. trachomatis* IgM MIF titre results <32 were not further investigated for any pathogens, thus the etiology of these cases remained unknown. In the case of weak seropositivity in clinically suspicious patients, a subsequent retesting 2-4 weeks later can be helpful so as to observe any changes in IgM titres.

Male infants appeared to be more vulnerable to RTI than females (ratio 1.3), and also to a severe infection requiring hospitalization (ratio 1.4), especially due to a *C. trachomatis*-infection (ratio 1.7), suggesting that males are more vulnerable to severe *C. trachomatis*-RTI. This is very different in infants with ON, where the male to female ratio was 0.9 for all ON, 1.0 for *C. trachomatis*-ON and 0.6 for severe ON requiring hospitalisation. Furthermore, the time of onset of the *C. trachomatis* related disease also differed: the median age of patients suffering from an early onset-type *C. trachomatis*-ON was 2 weeks, while it was 9 weeks in *C. trachomatis*-RTI cases. This is similar to previously reported data [12]. Only 6% of the RTI patients belonged to a younger age group than 4-12 weeks, which also corresponds to the clinical observations [13].

Compared to chlamydial ON, the rate of hospitalisation from RTI was high: 80 % (vs 6.7% of ON). This is due to the need for a more detailed diagnostic procedure, and for a more prolonged therapy and follow-up. Furthermore, in premature babies, the increased risk for developing respiratory distress syndrome (RDS) may also favour hospital admission [40].

Based on the positive IgM MIF results (titre ≥32), 50 of 262 infants were diagnosed with *C. trachomatis* infection, which means a 19.1% seroprevalence in this group. These prevalence data are far higher than the recently published 7% prevalence based on the direct PCR

detection of *C. trachomatis* DNA [44]. This discrepancy may be attributed to the different detection methods as specific IgM may persist up to 3 months in serum samples, while the chlamydial DNA rapidly disappears after an antibiotic treatment [47].

The clinical significance of *C. trachomatis* is supported by the observation that it has still been detected as the second most frequent respiratory pathogen after RSV of infants less than 6 months old in the Netherlands [44]. Detailed surveillance reports are still unavailable but *C. trachomatis* infection and testing should always be considered when viral etiology is excluded or less likely [48].

As the background for diagnosis, PCR detects the pathogen's DNA while serology detects the specific IgM due to an active immune response. The onset and quality of tests results may be influenced by several extrinsic/intrinsic factors, for example by any antibiotic therapy, steroid treatment, transfusion, plasmaferesis, an infective dose of *C. trachomatis*, the timing of sample collection etc.. However, in untreated cases with an intact immune system one can expect a positive PCR result first, followed by a massive IgM production.

Among the limiting factors of the study one should note that, due to the prior antibiotic treatment the testing of some relevant samples of nasopharynx or conjunctivae proved futile, which made the diagnosis of *C. trachomatis* infection somewhat less definitive.

The maternal cervical samples were also not available for a parallel *C. trachomatis* testing, although this investigation would have provided additional valuable data about the *C. trachomatis* prevalence in women, and it might also have provided a possible explanation of the potential source of infection in the seronegative or weak seroreactive infants. Furthermore, it would have been informative to know the distribution of the maternal chlamydial genotypes as in a former research we had confirmed that certain genotypes are more likely to cause *C. trachomatis* ON than others.

The current prenatal screening system does not include *C. trachomatis* maternal tests, moreover, we have no prevalence data either of pregnant women or of infected neonates. According to WHO reports in 2012 the prevalence of *C. trachomatis* was estimated to be 4.2% among fertile women globally, while the it was found 7.9% in 2003, and 8% in 2007 among Hungarian young women, respectively [49,50,51].

Calculating with the range of a previously estimated 4-8% maternal prevalence and with 90 000-95 000 deliveries per year in Hungary, we can safely assume that about 4 000-8 000 pregnant women may be infected by *C. trachomatis.* Being aware of the transmission rate, as many as 2000-5000 newborns can be colonised resulting in a symptomatic ON of 700-2800

cases, while RTI of 200-1100 cases. This estimated high number of patients indicate focused screening and therapeutic efforts to reduce the number of *C. trachomatis* infected infants, and the routine screening of pregnant women should be implemented in order to potentially decrease the rate of vertical transmission.

Neonatal STIs are still often undetected, misdiagnosed or treated inappropriately, although modern laboratory methods are available both for prenatal and postnatal diagnostical purposes. Our data demonstrate that *C. trachomatis* is still a leading pathogen among respiratory neonatal infections in Hungary. Chlamydial etiology was found in one fifth of the newborns with RTI, as stated in our first report of this kind in Hungary so far.

Summarizing our results we would like to point out that serology may be a useful and reliable alternative diagnostic test when no respiratory samples are taken prior antibiotic treatment for PCR. The parallel testing of clinical samples for the most frequent respiratory pathogens enable a targeted therapy and the recognition of a potentially mixed infection.

Besides prenatal screening as a potential tool of prevention, neonatologists and neonatal nurses should also be aware of the risks and the prevalence of *C. trachomatis* infection and about the possibilities of correct sampling and laboratory diagnosis. They have a crucial role at the early recognition of ocular and respiratory chlamydial infections.

Diagnosed *C. trachomatis* infections of neonates, however, may not only contribute to better chances of recovery of the patient itself but also indicate further STI screenings. It is not uncommon that this laboratory diagnosis is the only indirect sign of the latent chlamydial infection of the mother and of her sexual partner(s). As a late sequelae of ascending, unrecognized and untreated maternal infections chronic pelvic inflammation may develop in 2-5% of mothers, resulting in subsequent infertility disallowing further pregnancies [52].

5.2 Detection of *C. trachomatis* specific immune response among verified LGV patients

Lymphogranuloma venereum, an STI caused by *C. trachomatis* L 1-3 serotypes is endemic in tropical regions and there had been only imported cases diagnosed in Europe before 2003. During 2003-2004, however, an LGV outbreak was recognized in Rotterdam and increasing numbers of cases have been reported throughout Western-Europe since then [16].

There were almost 1800 LGV cases reported from Europe in 2015, while the majority, 87% of them still originated from three countries, namely France, the Netherlands and the United Kingdom [53]. Due to several facts, such as limited access to molecular biological diagnostic

possibilities; the high rate of symptomless carriers; and the difficulties around contact tracing, this condition must be well underrecognized and underreported, however, the reported number of cases still exceeds those reported in 2014 by 26% [53].

Most LGV patients belong to a high-risk MSM population, who may also be frequently infected with other STI agents due to the multiple number of casual partners, ignoring safe sexual practises, sharing sex toys etc. Nearly 70% of LGV patients with a known serostatus are human immundeficiency virus (HIV) seropositive [16]. The age-groups of LGV patients show that the majority of cases (93%) are evenly distributed in patients older than 25 years [53]. We have observed this phenomenon also among the limited number of cases verified in Hungary. LGV only rarely affects heterosexual men or women, if so, this is transmitted to them by bisexual men, so called „bridging" persons [54].

The leading clinical symptom of LGV is proctitis, an inflammation of the rectal mucosa, i.e. of the distal 15 cm portion of the colon, which gives rise to several differential diagnostical problems. The most frequent bacterial STI agents causing infectious proctitis are *N. gonorrhoeae* (30%), *C. trachomatis* (19%) and *T. pallidum* (2%) [55]. Co-infections are present in about 10% of the patients but in the absence of a targeted venereological examination and anamnestic data, the risk factors of exposition are not necessarily revealed during a proctological investigation. As the symptoms of proctitis are mainly associated with inflammatory bowel diseases (IBD), such as Crohn's disease or ulcerative colitis, the rectal infections caused by LGV or other STI agents are commonly misdiagnosed. There are several case reports of LGV patients having been misdiagnosed with IBD, and we have also experienced this false diagnosis in minimum 10% of our verified cases in Hungary [56].

Unrecognized, untreated acute LGV is a dangerous condition, which may turn into a chronic disease characterised by colorectal fistulas, granulomas, ulcerations, the development of fibrotic tissue and a subsequent lymphatic obstruction, while the patient itself can infect further persons via unsafe sexual contact [55].

Chlamydial seropositivity indicating this invasive infection is an alarming sign not only for acute LGV cases but also for chronic ones representing even more differential-diagnostical problems. Moreover, the testing of serum samples enables not only the detection of *C. trachomatis*-antibodies, but also a parallel screening for frequent concomitant agents such as HIV, hepatitis B virus (HBV), hepatitis C virus (HCV) and *Treponema pallidum*.

The other important field of serological surveillance covers the screening for previous infections in a given population and a risk-assessment thereby; and as such, it facilitates to estimate the risk of being infected with LGV among heterosexual persons [54].

The number of verified LGV cases in Hungary has shown an increasing tendency since 2012, although the verified number of 53 LGV cases (as per our own laboratory data) between October 2012 and December 2017 does not necessarily reflect the actual number of patients for the various reasons detailed above.

Immunoblot assays performed with species-specific chlamydial antigens are reported highly sensitive and specific, therefore they may serve as a reliable serological tool of the presumptive diagnosis of LGV [57]. Moreover, they can be applied on a single serum specimen, sent in sporadically to the laboratory, making it a perfect choice from practical point of view too [17]. A robust anti-MOMP IgA reactivity, however, can not only be associated with a symptomatic LGV, but it is also present in 75% of asymptomatic LGV patients, thus providing a valuable diagnostic tool [21].

High levels of specific anti-chlamydial IgA and IgG in themselves may indicate the necessity of a relevant anogenital or inguinal sampling for a definite diagnosis, aiming the detection of *C. trachomatis* DNA and further genotyping of positive samples.

Negative results of immunoblot tests do not exclude LGV as an etiology, since the sampling may occur in a very early, seronegative phase of the infection, while the positive results referring to an acute (or a previous) infection serve as a good predictor of a chlamydial involvement until the molecular biological tests provide more definitive information.

The analysis of the *C. trachomatis* ELISA tests confirmed that all the samples of the tested LGV patients yielded positive IgA and IgG results, while we found a high IgA COI $\geq$ 2.0 in the majority (86%) of the patients. Samples (N=5) with IgA COI values<2.0 however were found strong positive for specific IgG of a COI $\geq$ 1.9. The median values of IgA and IgG ELISA results were several folds higher in the group of LGV patients compared to those of infertile patients, which proved to be a significant difference.

All the serum samples of verified LGV patients (N=36) were strongly positive for both IgA and IgG with the immunoblot method, similarly to reports applying other serological assays (ELISA, immuno-fluorescence etc.) supporting the invasive nature of this infection [20]. When compared to results of those infected by *C. trachomatis* D-K serotypes we did not find any difference in the reactivity pattern, while there was a much more robust intensity observed in all LGV patients due to the extremely high levels of antibodies.

It would have been interesting to extend the study to a serological analysis of a male study group suffering from proctitis caused by *C. trachomatis* D-K serotypes, alas, we have only a few samples of verified cases until now. Moreover, one can never exclude a previous, undiagnosed LGV and its consequent effect influencing the level of anti-chlamydial antibodies in MSM.

The currently available IUSTI guidelines recommend the screening of every MSM practising receptive anal sexual contact for the previous 6 months for an anorectal *C. trachomatis* infection. Subsequently, positive patients should also be tested for LGV. Testing for other STIs, such as HIV, hepatitis B and C, as well as for syphilis and gonorrhoea should be also offered for the patients due to the high rate of co-infections [17].

We suggest that *C. trachomatis* serology should also be selectively added to the STI screening in symptomatic patients should their clinical signs and anamnestic background raise the possibility of an LGV infection. Serology may be useful for recognizing not only acute cases but also chronic untreated conditions mimicking IBD. Our results strongly suggest the diagnostic utility of immunoblotting in this condition. To our best knowledge this is the first time that immunoblotting has been applied as an alternative supportive tool for a presumptive LGV diagnosis.

5.3. Determination of *T. pallidum* seroprevalence among pregnant women

Besides the classical venereological signs and symptoms of the infected mother, the most frequent pathologic manifestations of a syphilis infection are adverse pregnancy outcomes, such as miscarriage, stillbirth, preterm delivery, neonatal death and congenital syphilis.

The intensified antenatal screening program, launched by the WHO in 2007 resulted in halving the seropositive rates among pregnant women between 2008 and 2014 [58,59]. The varying distribution of estimated maternal syphilis across the various geographical regions of the WHO is among the lowest in Europe, with an estimated 3.3‰ prevalence in 2008 and 1,5‰ in 2012 [60]. This decreasing trend, which is due to more effective and extended intervention efforts, is also reflected by ECDC data, reporting decreased syphilis rates among women from 3.2 to 1.6 per 100.000 from 2005 to 2013, respectively [6].

Although the global syphilis surveillance data decreasing between 2008-2012 suggest a substantial progress towards the successful elimination of congenital syphilis, there are still many obstacles to overcome [30,60]. One should ease the access to prenatal screening and

prevention in early pregnancy for everyone as well as to adequate care and to follow-up when needed. It should be emphasized that the elimination of congenital syphilis will always depend on the actual surveillance strategies covering the population of reproductive age. An isolated screening protocol focusing exclusively on pregnant women can be regarded as only a suboptimal solution [30].

The 2.9‰ seroprevalence in Hungarian pregnant women corresponds well to the European data published by WHO, although it is almost double the calculated prevalence of 2012. Compared to the earlier data of other European countries this value is also higher than the reported 1.7‰ in Italy (2006-2007), but lower than the 5.5‰ prevalence in Bulgaria (2009-2013) [61,62]. In Ireland a syphilis prevalence ranging annually from 1.4 to 3.3‰ was recorded among pregnant women during a 6 year period from 2005 to 2010 [63].

Besides serology being the gold standard diagnostic laboratory method, direct detection techniques, such as dark field microscopy, PCR etc. can theoretically be also performed on material taken from the mucocutaneous lesions during the infectious stages of syphilis, such as primary chancres or condylomata lata. However, based on our direct laboratory experience it is extremely rare to receive such clinical specimens, and if so, they almost never come from pregnant women. Indeed, of these 148 seropositive patients in our series, not one single case had been tested this way.

Except for the early, seronegative phase of syphilis, serological tests have a crucial role in diagnostics. As we do not have any updated national recommendations, we chose a combination of RPR and TPHA tests as first-step methods for testing the prescreened maternal sera, allowing the parallel detection of both aspecific and specific antibodies [4,14]. Serological tests should not only confirm or exclude *T. pallidum* infection, but also should offer titrated reagin values to allow a proper serological follow-up after treatment, as the decrease of the titre suggests a successful healing, while an increase thereof is a probable marker of a reinfection [64,65].

Samples proved to be reactive to RPR and/or TPHA tests were further investigated by ELISA in the second step of the verification process. All the patients found IgG positive were interpreted as syphilis seropositive, and their infectivity, i.e. the risk of vertical transmission was assessed by the level of the titrated RPR.

Although the dynamic follow-up of reactive RPR of the repeated samples would be optimal, one can evaluate the possibility of infectivity even by an RPR titre of a single sample. Based on literature data, an RPR titre of 8 was chosen as the diagnostic cut-off, and we decided on a

1:16 or higher dilution RPR titre as an indicator of a probable recent, infectious syphilis, with an increased risk of a vertical transmission [14,32,39,66]. Indeed, the risk of transmission is correlated with the RPR titre, which is highest at the stage of symptomatic, primary syphilis with a transmission rate of 70%-100%, and 40% during the early latent phase (<1 year), and (still) 10% during late latent syphilis (>1 year) [64].

The subgrouping of the seropositives by RPR level indicated that at least 27% (40/148) of the confirmed syphilis seropositive pregnant women were most likely in the recent, infectious stage of syphilis. This does not even take into account the theoretically possible eventuality of very early syphilis cases, where the RPR is still low, but will rise over time if left untreated. Half of the infectious syphilis cases were identified among the youngest, most vulnerable group of pregnant women aged between 15 and 24 years. These data are similar to those of a UK surveillance study, revealing 25% of newly diagnosed syphilis among seropositive pregnant women in the period of 2010-2011 [67]. Although the risk of having a maternal infectious syphilis seems to decrease by age, it is remarkable that we have found 6 cases out of the 40 suffering from active, infectious syphilis in the age group of 35 years or above.

Despite the general advice to perform screening as early as possible in pregnancy, we confirmed almost half of the cases in their 2nd trimester, and one fifth as late as in the 3rd trimester. We do not know the reason for those instances of late testing, but the late presentation for the first antenatal care could most likely play a role, especially in multiparous and/or socio-economically deprived women, such as drug-users, homeless, etc [39].

Late treatment is not a rare phenomenon, since a UK surveillance study also reported that 18% of pregnant women with syphilis had not received the adequate treatment until their 3rd trimester due to socio-economic problems or lacking prenatal care [67]. Treatment for maternal syphilis less than 1 month before delivery always raises concerns, as it may be too late to prevent neonatal complications due to CDC case definition [68]. In these cases the infants should always be evaluated for congenital syphilis too.

In our series the majority (at least two-third) of seropositive women - similarly to the previously cited British survey - realized they had an infection only during the course of prenatal screening, facing the so-called „unexpected seropositivity”[67]. This underlines the crucial role of routine syphilis screening not only in order to prevent neonatal cases but also to reveal and to treat their own infection. In a Scandinavian study, even with a low seropositive rate of 2/10.000 it is still cost-beneficial to perform a routine screening [69].

In our series only a minority (13%) of the infected cases were diagnosed by venereologists due to contact tracing, while more than half of them (N=11) had RPR levels >8 suggesting infectious syphilis. Over a period of 4 years, only in the population serviced by our laboratory in Hungary, the lack of maternal screening would have missed 29 recent infectious cases of syphilis in pregnancy, i.e. at least 7 per year. Despite the limited information regarding seropositive patients we can conclude that the routine screening in Hungary at least revealed 72.5% of all pregnant patients at risk of transmitting *T. pallidum* to their fetuses.

A limiting factor that impaired the interpretation of the serological results was the lack of clinical data and that of serological follow-up, which would have helped to better determine the reasons of late screening, or no screening at all. In addition, the denominator of the number of women attending a venereologist for opportunistic screening is not known, which hampers the interpretation of these data obtained from women outside the screening protocol.

The testing of paired sera would have helped to decide between past or early acute infections by checking the actual changes of the initial RPR titres ≤8, as well as the response to therapy by an expected fourfold decrease of high initial RPR titres, or even a risk of reinfection among high-risk pregnant women.

Finally, in this surveillance study we do not have any feedback about the neonates' clinical and serological status, which hinders our efforts to make a firm conclusion on the full extent of the implementation of a generalized screening. For this reason we suggest a better controlled, generalized mandatory prenatal screening and the systematic obligatory reporting of positive cases in Hungary.

To our best knowledge this was the first study in Hungary aiming to determine the syphilis seroprevalence among pregnant women, as well as the demographic and epidemiological features of the seropositive mothers. The observed high rate of potentially infectious patients urges for a more effective screening strategy, which should include a more concentrated effort in focusing on the youngest age-group, as well as involving an increased number of women screened in the first trimester.

6. CONCLUSIONS

6.1. Determination of *C. trachomatis* seroprevalence among neonates suffering from RTI

a) A 19.1% *C. trachomatis* seroprevalence was found among symptomatic neonates suffering from RTI tested with a specific IgM MIF assay. The seroprevalence found corresponds to some earlier published data and may well reflect the actual risk of infection in the absence of prenatal screening. According to some reports, *C. trachomatis* may be the second most common RTI-pathogen in this age group second only to RSV-infections [44]. Summarizing our findings, a parallel RSV-testing of this patient group would be much more reasonable than the current clinical approach focusing on pertussis.

b) Our serological analysis revealed a median age of 9 weeks in this patient group (versus ON patients with a median age of 2 weeks), in correspondence with literature data. This condition is described as a so called „late-onset" RTI affecting most often neonates aged 2 months [13]. Due to the severity of RTI 80% of them required hospital care (vs 6.7% of ON patients). In spite of only a low number of seropositives, our results support the observation regarding the increased vulnerability of male infants towards RTIs, especially towards the more serious infections requiring hospitalization [70]. Male gender dominance was not present among ON patients.

c) As this age-group is partially unvaccinated against pertussis, we would like to emphasise that all neonates suffering from pertussis-like symptoms should be tested for *C. trachomatis* too. Based on our results there is a very low risk of being infected by *B. pertussis* compared to *C. trachomatis*, as we could not confirm any pertussis cases among the tested population during this long study period.

6.2. Detection C. *trachomatis* specific immune response among LGV patients

a) The ELISA and immunoblot results of the analysed LGV cases (N=36) in Hungary correspond to the literature data reporting elevated specific IgA and IgG production due to this invasive type of *C. trachomatis* infection. Especially the detection of a high IgA level can be an adequate predictor of an actual LGV. The ELISA test based intense anti-MOMP IgA reactivity published earlier was also observed on the immunoblot strips, first described by ourselves as having applied this laboratory method.

b) In our study we have compared the *C. trachomatis* ELISA IgA and IgG results, as well as the IgG immunoblot reactivity of the LGV study-group to those of a non-LGV patient group of infertile women suffering from an ascending type of *C. trachomatis* D-K infection. We have found that the samples of the LGV patients yielded significantly higher levels of IgA and of IgG than those of the infertile patients, characterised by a strong positive IgA response among LGV patients. The median ELISA COI values of both tested antibodies proved at least twice higher among LGV patients than in infertile patients.

c) Irrespectively from the chosen serological method, the strong positive IgA and IgG results may have a diagnostic impact as well, indicating further testing for a suggestive LGV. In case of large numbers of specimens we suggest that a *C. trachomatis* ELISA test be used. Having a low number of sporadic LGV cases, the use of immunoblot is more informative and cost-effective, even when testing a single sample for presumptive diagnostic purposes. As a valuable diagnostic tool it can raise awareness to the necessity of a targeted investigation of the relevant anogenital samples in asymptomatic or misdiagnosed LGV patients, especially as an ideal screening technique for IBD patients. Due to difficulties of contact tracing among MSM populations and to frequent co-infections, serological testing may serve not only as a diagnostic tool but it is also suitable for complex STI screening purposes.

6.3. Determination of *T. pallidum* seroprevalence among pregnant women

a) The 2.9‰ syphilis seroprevalence found in the pregnant population tested between 2013–2016 counts as an average value compared to the earlier published European seroprevalence data, but exceeds the 1.5‰ value of 2012 as estimated by the WHO.

b) We have found that at least 27% of the seropositive group (N=148) has a probable active, infectious syphilis, characterised by the highest risk (70-100%) of a vertical transmission. As one can count with the possibility of being tested in an early phase of infectious syphilis in patients having RPR titres≤8, its risk could not be excluded among them either.

c) We have found that high-risk, infectious syphilis occurred in every age-group, even among those older than 35 years, nevertheless, the youngest mothers of 15-24 years yielded the half of all cases with probable acute syphilis.

d) Instead of the recommended visit during the first trimester of the pregnancy, almost half of the 123 seropositive patients were diagnosed in the second, a fifth of them as late as in

the third trimester. The latter rate is highly alarming as any therapy given within one month of delivery is regarded inadequate, increasing the risk of a connatal syphilis.

e) The efficacy of a routine prenatal screening versus random venereological testing was supported by the fact that almost three quarter of the high risk pregnant women were diagnosed through the former. Consequently, the routine prenatal screening still has a very important role in revealing gestational syphilis and preventing congenital syphilis.

7. NOVEL SCIENTIFIC FINDINGS

1. The first investigation of seroepidemiological features of neonatal *C. trachomatis* infections in Hungary as well as its comparison to relevant international data. Based on the laboratory results, a further evaluation of the introduction of prenatal screening is to be considered.

2. *C. trachomatis* IgM-seroprevalence was found 19.1% among Hungarian neonates suffering from RTI. Pertussis-like neonatal syndromes should be always investigated for *C. trachomatis* as well.

3. The first application of immunoblot technique for a presumptive laboratory diagnosis of LGV patients. An elevated *C. trachomatis* IgA-level serves as a good serological predictor of LGV.

4. Significant differences of *C. trachomatis* IgA and IgG levels between male LGV patients and infertile women suffering from ascending *C. trachomatis* D-K infections.

5. The first analysis of syphilis seropositive pregnant women in Hungary focusing on age, gestational age, assessed infectivity, as well as on the role of mandatory screening. Comparison of found data to international survey results.

6. Syphilis seropositivity was found in 2.9‰ among tested pregnant Hungarian women between 2013-2016. 27% of all seropositive patients suffered from acute/recent infectious syphilis with an increased risk of vertical transmission. Half of infectious syphilis patients belonged to 15-24-year age group. Almost half of infectious syphilis patients were diagnosed in second trimester, while one fifth of them were as late as in the third trimester.

7. The results have clearly indicated the important role of a routine prenatal screening versus a random STI testing among pregnant women.

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