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

PhD Thesis

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CONTENT

1. INTRODUCTION

1.1. Bacterial STIs: main pathogens, epidemiological data

Based on 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 last year, which may still underrepresent the current epidemiological situation of Hungary [2]. 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 manifestating 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. Urogenitalis 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 (manifestating as endometritis, salpingitis, 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].

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% [8]. 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 (RTI) occurs at a lower rate, between 10-20% of them. Approximately half of the RTI cases are accompanied with the telltale sign of ON [9,10,11]. 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 [12].
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. The routine prenatal screening in Hungary currently does not include *C. trachomatis* maternal cervical tests. However, in accordance with the latest recommendations of CDC, all pregnant women aged <25 years and those at an increased risk for chlamydia should be screened not only at first prenatal visit but should be also rescreened during the third trimester [13]

1.2.1.2. Diagnostic relevance of the systemic immune response in neonates

Similarly to adults' uro/-anogenital infections the local inflammation in ON 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. It is a valuable diagnostic tool in supporting the chlamydial etiology [3,14].

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) or low respiratory tract specimen are recommended to test for the presence of *C. trachomatis* [13]. 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)

Lymphogranuloma venereum (LGV) is a sexually transmitted disease caused by *C. trachomatis* serotypes L1-3. 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 characterised by various, often non-specific symptoms. 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 (MSM) 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 [15]. Asymptomatic LGV carriers may also serve as an infectious source of the disease 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

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 [17]. A strong IgA and IgG seropositivity may provide a presumptive diagnosis completing the first-line approaches focusing on the detection of LGV biovar specific DNA. 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 [3,18,19].

1.2.2.2. Possibilities of laboratory diagnostics in LGV

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 [16].
Besides direct detection techniques, serum samples of LGV patients are also suitable for the testing of specific IgA and IgG applying various serological assays [19]. 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 IUSTI [16].

1.3. *T. 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 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 [20]. Syphilis is a chronic invasive infection caused by *T. pallidum* subsp. *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 [21].

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; while due to the variable, often atypical presentations of secondary syphilitic mucosal or skin lesions, a misdiagnosis is a common occurrence [22]. 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 [23]. 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 [23]. Despite the massive immune response no protective immunity exists in humans against *T. pallidum* [24]. The follow-up of the changes of reagin 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 play a crucial role in establishing the actual stage of syphilis in patients [4,23].

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 serious adverse birth outcomes due to transplacental transmission, and threatening the health of the mothers themselves in untreated cases [25]. Women suffering from syphilis may infect their fetuses any time during their pregnancy. 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. The distribution of the potential fetal and neonatal consequences depends on the actual level of maternal infectivity [26].

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 [27]. 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 [28]. 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 (RPR, VDRL, 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). Specific IgG is the serological marker of syphilis seroprevalence, i. e. the persistant 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 [4,23].

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 automatisation of the tests and the estimated costs thereof. The aspecific tests make 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 [29]. Treponemal tests are suitable for the total or separated detection of specific IgG and IgM antibodies but they are not helpful in assessing the disease activity and following treatment outcome. It should be emphasized that all the laboratory results should be assessed together with the anamnestic data and the actual clinical signs and symptoms [22,30].

2. AIMS

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

Our aims were the following:

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 of the seropositive patient group, i.e. the distribution of age, gender and the need for hospitalisation compared to patients suffering from ophthalmia neonatorum;

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

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

Our aims were the following:

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 *C. trachomatis* D-K serotypes.

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

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

Our aims were the following:

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 recommandation 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. 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. Due to clinically similar symptoms the testing of all neonates in the study group was also indicated for pertussis.

3.1.2. Detection of *Chlamydia trachomatis* specific IgM

A micro-immunofluorescence (MIF) test (Focus, Cypress, USA) was chosen used in accordance with the manufacturer's instructions to detect the *C. trachomatis*-specific IgM in these serum samples. The cut-off was defined at a serum dilution rate of 1:32 considered to be diagnostic for the infection [17]. Based on these results, the patients were divided into 2 main groups. Samples of seropositive patients showed an intense, bright-green fluorescence under UV-microscope due to the binding of fluoresceine-labeled antihuman-IgM to the chlamydial antigen-human IgM complexes. All the other (non-reactive, indeterminate or weak reactive) cases were interpreted seronegative.

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 *Chlamydia 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. 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 immunoblot tests

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 enables the detection of reactivity against the following antigens: some genus-specific antigens, such as chlamydial LPS and HSP 60 heat shock-protein (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 [31,32]. 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 for the presence of specific *T. pallidum* IgG/IgM antibodies between 2013-2016. All the samples showing reactivity (N=527) were submitted for further verification of *T. pallidum* infection to our laboratory.

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

Confirmation process included a stepwise performation of combined serological assays of different principle. First, the combination of a titrated Rapid Plasma Reagin (RPR) test (Omega Diagnostics, Alva, Scotland) and a qualitative Treponema Pallidum Haemagglutination Assay (TPHA) (Trinity BioTech, Bray, Ireland) was applied. When both of these tests were found negative, the samples were judged seronegative 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 [13,29,33]. (Based on diluted RPR results (negative; 1≤ 8; or >8) the seropositive patients were divided into 3 groups. 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.

4. RESULTS

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

4.1.1. Serological resulst, 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). 50 of 262 patients (19.1%) were found unequivocally seropositive when tested in the chosen dilution (diagnostic cut-off of 1:32). Altogether 212 infants were interpreted as seronegative. According to these results seroprevalence was found 19.1% (50/262) in this study group.

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). 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, i.e. *Bordetella pertussis* as an etiological agent could be excluded from a differential diagnostical point of view.

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), as 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 *Chlamydia trachomatis* IgA and IgG. The ELISA results were 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.

4.2.3. Comparison of serological results

The non-LGV study group consisted of *C. trachomatis* seropositive infertile women (median age: 36). They 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 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.

As all the samples of the infertile women were found positive for *C. trachomatis* IgG showing reactivity by immunoblot 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.

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:

1) RPR-negative cases indicative of a past infection were found in 53 women (36%);
2) weakly reactive RPR (titers≤1:8), referring to as either a past or an early acute infection, was observed in 55 women (37%).
3) strong RPR reactivity (titers>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. 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. 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. Altogether 29 (72.5%) of all patients suffering from a presumably acute, infectious syphilis were diagnosed due to prenatal screening.

5. DISCUSSION

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

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 CT infection in newborns. 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 [12,34]. It can cause a diagnostic challenge, especially in unvaccinated young infants, but the real risk of pertussis depends on the actual epidemiological situation, principally when similar cases are accumulating and/or no vaccination can be assumed among the contacts.

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) for the presence of the pathogen [13]. In this study most of the infants had already received some antibiotic treatment, rendering the negative results from PCR testing unreliable. In the absence of relevant respiratory samples the specific IgM response can be detected with serological assays as a supportive test [3,14]. 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 CT-infection (ratio 1.7), suggesting that males are more vulnerable to severe CT-RTI. This is very different in infants with ON, where the male to female ratio was 0.9 for all ON, 1.0 for CT-ON and 0.6 for severe ON requiring hospitalisation. Furthermore, the time of onset of the CT related disease also differed: the median age of patients suffering from an early onset-type CT-ON was 2 weeks, while it was 9 weeks in CT-RTI cases. This is similar to previously reported data [11]. 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.

Based on the positive IgM MIF results (titer ≥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 [35]. 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 [36]. 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 (35).

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. Calculating with the range of an 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. 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).

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 [15]. Most LGV-patients belong to a high-risk MSM (men who have sex with men) population, who may also be frequently infected with other STI agents. Nearly 70% of LGV patients with a known serostatus are HIV seropositive [15]. There were almost 1800 LGV cases reported from Europe in 2015, reflecting that this condition must be well underreported [37]. LGV only rarely affects heterosexual men or women, if so, this is transmitted to them by bisexual men, so called „bridging" persons [38].

The leading clinical symptom of LGV is proctitis, 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%) [39]. 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 [40]. 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 [39]. Chlamydial seropositivity concomiting this invasive infection is an alarming sign not only for acute LGV cases but also for chronic ones representing even more differential-diagnostical problems.

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. 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. 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 [19]. When compared the results of LGV patients 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 stronger intensity observed in all LGV patients due to the extremely high levels of antibodies. 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 [18,41]. 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 [16].

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

The intensified antenatal screening program, launched by the WHO in 2007 resulted in halving the seropositive rates among pregnant women between 2008 and 2014 [42,43]. 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 [44]. 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 [27,44]. 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 [27].

The 2.9‰ seroprevalence in Hungarian pregnant women corresponds well to the European data published by WHO. 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) [45,46]. 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 [47].

Except for the early, seronegative phase of syphilis, serological tests have a crucial role in diagnostics. According to international guidelines 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 [4,13]. Samples proved to be reactive to RPR and/or TPHA tests were further investigated by ELISA. 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 [29,33,48]. The risk of transmission is between 70%-100% at the stage of symptomatic, primary syphilis, 40% during the early latent phase (<1 year), and (still) 10% during late latent syphilis (>1 year) [49]. 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. Half of the infectious syphilis cases were identified among the youngest women aged between 15 and 24 years. 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 [33]. 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 [50]. In our series the majority (at least two-third) of seropositive women realized they had an infection only during the course of prenatal screening, facing the so-called „unexpected seropositivity". 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 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.

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

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 [35]. 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) this condition is described as a so called „late-onset" RTI affecting most often neonates aged 2 months. It was supported also by our serological analysis in this patient group with a median age of 9 weeks (versus ON patients with a median age of 2 weeks). 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 [51]. 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.

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

a) the ELISA and immunoblot results of the verified LGV cases in Hungary corresponds to the literature data reporting elevated specific IgA and IgG production due to this invasive type of 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 method.

b) 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 form an ascending type of *Chlamydia 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 patiens.

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 popultions 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.

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) 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$\leq$8, its risk could not be excluded among them either.

c) we 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 syphils and preventing congenital syphilis.

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2. Luca Kormos, **Eszter Balla**, Janos Szlavik et al. Neurosyphilis in HIV-infected patients - differential diagnostics. IUSTI Europe, Budapest, 15-17. Sept 2016
3. Nikolett Csizmár, Dominika Binder, Laura Bense, Gyula Tálosi, Fruzsina Petrovay, **Eszter Balla**, Edit Kelemen: Sexually transmitted agents causing atypical respiratory tract infection in neonates. IUSTI Europe, Budapest, 15-17. Sept 2016. (POSTER)
4. **Eszter Balla**, Viktória Várkonyi: High rate of infectious syphilis among seropositive pregnant women in Hungary, 2016; IUSTI Europe, Helsinki, 31. Aug-1. Sept 2017. (POSTER)
5. **Eszter Balla**, Fruzsina Petrovay, Gilbert G. G. Donders: Seroprevalence of anti-*Chlamydia trachomatis* IgM in neonatal respiratory tract infections in Hungary, 2008-2016. 2nd ISIDOG Congress, Vienna, 26-29.Oct. 2017. (POSTER)
6. **Eszter Balla**, Fruzsina Petrovay, Tímea Erdősi, Gilbert G. G. Donders: Distribution of conjunctival *Chlamydia trachomatis* genotypes in ophthalmia neonatorum in Hungary, 2008-2016. 2nd ISIDOG Congress, Vienna, 26-29.Oct. 2017. (POSTER)
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