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Incidence of congenital toxoplasmosis estimated by neonatal screening: relevance of diagnostic confirmation in asymptomatic newborn infants

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SUMMARY

Congenital toxoplasmosis is rarely identified by routine clinical examination. The aim of this study was to estimate the incidence of the disease in the region of Ribeirão Preto, south-eastern Brazil. A definitive diagnosis was made on the basis of the persistence of anti-*Toxoplasma* IgG antibodies beyond 1 year of age. Blood samples obtained from 15 162 neonates and adsorbed onto filter paper were tested for anti-*Toxoplasma* IgM antibodies. Fifteen samples gave positive results. A definitive diagnosis was confirmed in five of the 13 infants (38·5%) who completed follow-up. These five infants presented with serum IgM and/or IgA antibodies, and clinical abnormalities. Disease incidence was estimated to be 3·3/10 000 (95% CI 1·0–7·7), indicating the need for preventive measures. Neonatal screening is feasible, but screening tests with a better performance are required; positive screening results must be carefully confirmed.

INTRODUCTION

Congenital toxoplasmosis (CT) is characterized by a broad spectrum of clinical manifestations at birth, including varying degrees of neurological, ophthalmological and systemic involvement [1]. Although most newborns are asymptomatic [2], up to 85% may develop visual disturbances within the first two decades of life, and up to 55% will show neurological abnormalities [3, 4]. Several lines of evidence suggest that the early institution of appropriate treatment may reduce the frequency of late sequelae [1, 5].

The seroprevalence of toxoplasmosis in pregnant women and the incidence of CT are well known in various regions, particularly in European countries [6–11]. This knowledge has allowed adequate assessment of the burden of the disease and the planning of preventive measures.

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In Brazil, a large country characterized by heterogenous socio-economic conditions and cultural and hygiene-nutritional habits, studies involving a limited number of pregnant women from different communities have reported high rates of toxoplasmosis seroprevalence, ranging from 59·8 to 74·5% [12–16]. However, there have been few investigations that identify the real incidence of CT in different regions of Brazil. Recent reports have estimated that the incidence of CT ranges from 3·3 to 19·6/10 000 live newborns [17–19]. In these investigations, confirmation of the disease was obtained by the detection of anti-*Toxoplasma* IgM antibodies in the newborn. Although this detection has been used for definitive diagnosis [1, 5, 20], its use generally requires performing confirmatory testing. A definitive diagnosis would also be possible by the direct detection of the parasite in clinical samples, by demonstrating the persistence of anti-*Toxoplasma* IgG antibodies beyond 1 year of age and/or by the detection of anti-*Toxoplasma* IgA in the first 6 months of life [1, 5, 20].

The region of Ribeirão Preto, located in the state of São Paulo, south-eastern Brazil, is characterized by a hot climate, favouring the survival of *Toxoplasma* oocytes in the soil. The seroprevalence of toxoplasmosis in pregnant women in this region was estimated to be 61% in 1999 [12]. These data include the population of this area in the group at high risk for CT according to the model proposed by Frenkel [21].

Considering the lack of information regarding the incidence of CT in the Ribeirão Preto region, the present study aims to estimate the incidence of the disease through neonatal screening, using as confirmatory criteria the persistence of anti-*Toxoplasma* IgG antibodies beyond 1 year of age and/or the presence of elevated titres after termination of treatment. In addition, clinical and laboratory findings for the identified infants are described.

METHODS

Study population

As part of the obligatory neonatal screening programme for phenylketonuria, congenital hypothyroidism and haemoglobinopathies, the Screening Laboratory of the University Hospital of the Faculty of Medicine of Ribeirão Preto of the University of São Paulo (HCFMRP–USP) receives all blood samples adsorbed onto filter paper cards collected within the public health system of the 25 towns in the region of Ribeirão Preto. These samples account for ~90% of the 17 000 live newborns delivered per year in the region. All 15 162 children whose filter-paper samples were sent to this laboratory in 2001 were included in the present study.

The study was approved by the Research Ethics Committee of HCFMRP–USP. Prior to the study, the team responsible for sample collection in the 25 towns in the region received instruction regarding the characteristics of the investigation. Information concerning the study was provided through informative posters and leaflets distributed to the families at the time of sample collection. Written, informed consent for additional assessment and follow-up was obtained from the guardians responsible for those children who tested positive on neonatal screening.

Neonatal screening

Blood samples adsorbed onto filter paper were collected in hospitals and health centres throughout the region by trained personnel, using the standard technique of skin puncture in the calcaneal area. Samples were generally obtained from children aged up to 16 days, and stored for a maximum period of 10 days at room temperature. A 3-mm diameter disc was punched from each filter paper card, eluted and analysed for anti-*Toxoplasma* IgM by a commercially available, fluorometric, enzyme immunocapture assay (Neonatal *Toxoplasma gondii* IgM FEIA®, Labsystems, Helsinki, Finland) according to the manufacturer's instructions [22]. Samples showing an optical density $\geqslant 80\%$ of the mean obtained for borderline controls were considered positive. Positive results were confirmed by repeating the assay using two aliquots from the same sample.

Complementary assessment of mother and infant

Children who had confirmed positive results on neonatal screening for CT were invited for diagnostic confirmation. Peripheral blood was collected from the mothers and their infants for confirmatory serological tests. Serum obtained from the child was tested using a double-sandwich, enzyme immunocapture assay for the qualitative detection of anti-*Toxoplasma* IgM and IgA antibodies (Platelia Toxo®, Sanofi Pasteur, Marnes-la-Coquette, France) according to the manufacturer's instructions. Samples showing optical densities $\geqslant 80\%$ of the control cut-off provided by the manufacturer were considered positive. Maternal and infant serum samples were also submitted to an indirect fluorescent antibody test (IFA) for the quantitative detection of anti-*Toxoplasma* IgG and IgM. Briefly, serum was diluted successively up to a maximum dilution of 1 in 4000 and added to slides containing fixed *Toxoplasma* antigens. The slides were incubated with fluorescein-conjugated human immunoglobulin anti-IgG or IgM. The antibody titre was defined as the highest serum dilution at which fluorescence was observed.

In addition, these children were submitted to a complete clinical examination, ophthalmological assessment and cranial ultrasonography and/or computerized tomography. Consequent to any alteration identified during the initial clinical assessment, the children were tested for liver function, complete blood count, cerebrospinal fluid analysis and instrumental auditory evaluation (auditory screening). The children were also screened for congenital cytomegalovirus infection (detection of viral DNA by polymerase chain reaction) [23], syphilis (VDRL), and rubella

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(haemagglutination inhibition test). None were infected by these agents.

Follow-up and diagnostic criteria

All infants identified by neonatal screening were followed up until their infection status had been established. Assessments included the detection of anti-*Toxoplasma* IgG antibodies by IFA every 2–4 months. A diagnosis of CT was confirmed on the basis of persistence of specific IgG antibodies beyond 12 months of age, or the presence of elevated titres after termination of the antiparasite treatment. Children with decreasing IgG titres and who became anti-*Toxoplasma* IgG negative were considered to be non-infected. Treatment was provided based on current recommendations [1, 5] with sulphadiazine, pyrimethamine, folinic acid and spiramycin being provided free of charge.

Data analysis

Assuming that the incidence of CT would be 1/3000 births, the sample size necessary to estimate the incidence of infection with a 95% confidence level and a precision of 0·03% would be 12639. The incidence was estimated based on the number of children showing CT, using the binomial exact 95% confidence interval (CI) for descriptive studies employing random samples. Calculations were performed using the Epi-Info 6 program (CDC, Atlanta, GA, USA, November 1993). The age at which infants became anti-*Toxoplasma* IgG negative was estimated as the midpoint of the interval between the last positive anti-*Toxoplasma* IgG test and the first negative test.

RESULTS

Anti-*Toxoplasma* IgM was initially detected in samples from 101 (0·67%) of the 15162 infants included in the study. This result was confirmed in 15 infants (14·85%), 10 (66·7%) being males. The Figure summarizes the results of the initial evaluation and the follow-up.

Most infants (73·3%) were from urban areas. Birth weight ranged from 2060 to 3790 g (median 3120 g), and gestational age ranged from 32 to 41 weeks (median 39·5 weeks). The median age of the 14 infants evaluated during complementary assessment was 94·5 days (2–338). All mothers reported regular prenatal follow-up. However, only two mothers (of infant

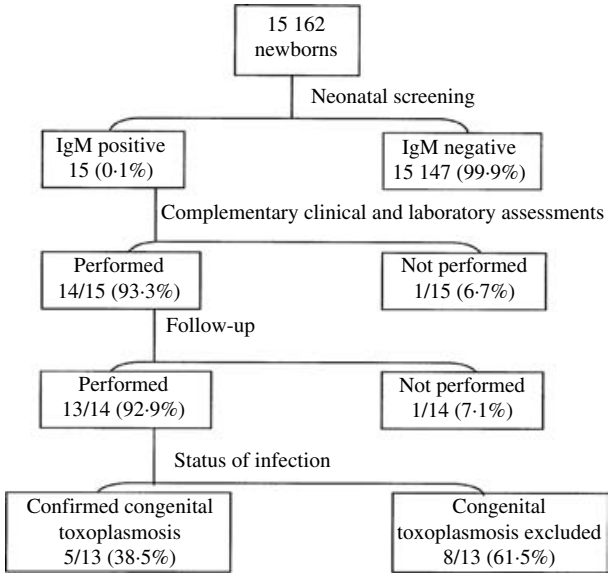

Fig. Neonatal screening for toxoplasmosis in children from the region of Ribeirão Preto, São Paulo, south-eastern Brazil.

nos. 6 and 12) were diagnosed with probable asymptomatic gestational toxoplasmosis due to detection of anti-*Toxoplasma* IgM in the serum at 26 weeks gestation, and to seroconversion at 31 weeks respectively. In the first mother, detection of genomic fractions of the parasite in the amniotic fluid by polymerase chain reaction (PCR) gave a negative result. The use of spiramycin until the end of pregnancy was prescribed in both cases, but only the second mother used the medication regularly.

With respect to the initial complementary laboratory evaluation, anti-*Toxoplasma* IgG at titres $\geqslant 1$ in 4000 were detected in serum samples from all 14 mothers by IFA, with no identification of IgM. Except for one child, initially evaluated at 11 months of age, IFA detected IgG antibodies in the remaining 13 infants, titres ranging from 1 in 256 to >1 in 4000; IgM was not detected. Enzyme immunoassays for the detection of serum anti-*Toxoplasma* IgA and IgM were positive in six of the 14 infants (42·9%), five infants being positive for IgM and IgA, and one infant being positive for IgA and negative for IgM.

Routine clinical examination showed signs and symptoms suggestive of congenital infection in two of the 14 infants (14·3%). One infant was not evaluated further. Complementary assessment revealed clinical abnormalities in five of the 13 remaining infants (Table). None of the 13 infants was diagnosed with auditory deficiency. All five symptomatic infants were

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Table. *Complementary clinical and radiological evaluations of five children*

Child (age in days at first evaluation)	Routine clinical examination	Positive findings on complementary assessment
No. 2 (110)	Normal	Macular scars due to chorioretinitis
No. 3 (104)	Normal	Temporal scars due to chorioretinitis, strabismus. Compensated moderate hydrocephalus. Elevated AST and ALT
No. 7 (98)	Hepatosplenomegaly, petechiae, ascites, jaundice	Turbid vitreous humor with active chorioretinitis in the left eye, strabismus. Parenchymatous calcifications
No. 8 (67)	Normal	Macular scars due to chorioretinitis, strabismus. Cranial US: images suggestive of previous haemorrhage. Cranial CT: prominence of the supratentorial ventricles. Elevated CSF proteins
No. 12 (2)	Hepatosplenomegaly, jaundice, microcephaly	Turbid vitreous humor with active chorioretinitis. Cerebral and cerebellar calcifications. Elevated CSF proteins. Elevated AST

US, Ultrasound; CT, computerized tomography; CSF, cerebrospinal fluid; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

treated for 1 year. Four of the eight asymptomatic infants received treatment for periods ranging from 15 days to 2 months, followed by withdrawal of the medication after a documented decline greater than 10 dilutions in anti-*Toxoplasma* IgG antibody titres.

The 13 infants were followed up for a mean period of 15·9 months (12·6–19·2 months). Five infants fulfilled the confirmatory criteria for CT: four of them (infant nos. 2, 3, 7, 8) maintained positive IgG titres at 1 year of age, and the other (infant no. 12) was seronegative at 12 months of age, but had a sudden rise in anti-*Toxoplasma* IgG titres after cessation of therapy. These five infants presented abnormal clinical findings on complementary assessment, and the presence of IgA and/or IgM in peripheral blood on first evaluation. The remaining eight infants were considered to be non-infected. In these infants, median age of becoming anti-*Toxoplasma* IgG antibody negative was 10·2 months (5·8–12·8 months), and no signs or symptoms attributable to CT or positive serological IgA or IgM tests were observed.

Estimation of the incidence of CT

Considering that five infants with confirmed CT were identified from a total of 15 162 infants submitted to neonatal screening, the incidence of the disease in the population studied was estimated to be 3·3/10 000 infants (95% CI 1·0–7·7). Assuming that the two infants who did not complete the study were also infected (both showed elevated quantitative results on neonatal screening, and one exhibited IgA and IgM antibodies in the peripheral blood), the incidence would be 4·6/10 000 infants (95% CI 1·9–9·5).

DISCUSSION

Based on the results of the present study, the incidence of CT in the Ribeirão Preto region is estimated to be 3·3 (95% CI 1–7·7) cases per 10 000 births. While this rate is similar to that reported in various Brazilian regions (3·3, 95% CI 2·5–4·4) [17] and in southern Brazil (8, 95% CI 0·2–44·5) [19] and to that found in Norway (3, 95% CI 1·5–5·5) [7], Denmark (3, 95% CI 2–4·4) [9], Slovenia (5·1, 95% CI 2·6–9·2) [11] and in Poland (5·5, 95% CI 3–9) [24], it is apparently lower than in Rio de Janeiro (19·6, 95% CI 6·4–45·7) [18], Switzerland (7·2, 95% CI 4·5–11·1) [25], Belgium (9·6, 95% CI 5·8–14·8) [26] and France (~10) [8]. However, the incidence of CT reported for the North-American region of New England [2], Sweden [10] and Hungary [6] is <1 case per 10 000 infants. These findings perhaps suggest epidemiological differences in infection in distinct localities, but the methodological peculiarities of the different study designs should be considered.

One advantage of the present study design is that the occurrence of CT in infants identified by neonatal screening was confirmed using a criterion considered to be a true indicator of the presence or absence of the disease [1, 20], but that has been applied in only a few previous studies [9, 10, 25]. Studies employing serological evidence of gestational maternal

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seroconversion [9, 10], in addition to the detection of IgM and/or IgA in newborns, are potentially more likely to estimate the true incidence of CT because they are not limited by the performance of neonatal screening, which may not detect some of the affected newborns [9].

In the present study, the use of a commercially available, fluorometric, IgM enzyme immunocapture assay developed for the detection of anti-*Toxoplasma* IgM in blood samples adsorbed onto filter paper was chosen because more sensitive tests were not avaliable [27]. The retest rate of 0·67% (101/15 162) obtained after the initial test for detection of anti-*Toxoplasma* IgM in dried blood samples was similar to the frequency of 0·5% reported by others [17]. This figure may have been elevated by the low cut-off level used in the screening tests, established to assure a high detection rate.

Even though FEIA performs well compared to similar assays conducted by experienced laboratories [22], there are no studies designed to evaluate its sensitivity and specificity properly, particularly when a definite diagnosis of CT is considered. Previous studies have reported sensitivities varying from 77·8 to 100% for IgM detection tests performed on neonatal dried blood samples [9, 10, 24], similarly to what has been shown for assays performed on blood [27]. In addition, IgM may not be present at birth in infected children, due to various immunological mechanisms [1, 28, 29]. Thus, supposing that the sensitivity of the IgM test did not reach 100%, it is possible that not all infected infants were identified, and that the incidence of the disease may be underestimated.

On the other hand, in the present study, 61·5% of the confirmed positive neonatal screening results were false-positive. Other investigators, using the same assay and diagnostic criteria reported 78·6% [10] and 91% [17] false-positive results. A high proportion of false-positive results is generally accepted for screening tests, especially in the case of populations with a low prevalence of the disease [30]. However, an elevated rate of false-positive results leads to a marked increase in screening costs, and generates anxiety and family issues due to the need for complex investigation and medical follow-up. In addition, emphasizing the need to follow IgG titres during and after treatment and to take the occurrence of serological rebounds into account for definitive diagnosis, it must be observed that infant no. 12, who was seronegative at 12 months of age, showed a sudden rise in anti-*Toxoplasma* IgG titres after cessation of therapy.

All infants with confirmed CT identified in this study had ophthalmological impairment on initial assessment, and 80% also presented neurological abnormalities. These findings contrast with those reported in other screening studies in which only 11–33% of infants presented such abnormalities, and 4–29%, neurological changes [2, 9, 10, 17, 24]. Even in studies evaluating mostly symptomatic infants, ~60–80% presented with ophthalmological and/or neurological impairment [31]. One fact that may explain the higher percentage of symptomatic infants in the present study is the age at which the first complementary examination was performed, i.e. a median age of 94·5 days. In contrast, other screening studies examined infants within the first 6 weeks of life [2, 9, 10, 17, 24]. Since ophthalmological impairment may not be clinically apparent for months or even years, ocular lesions may have occurred during the first months of life. However, even studies which have followed infants with CT up to 1–6 years of age have not reported the presence of ocular lesions in all of them [32]. Some investigators have tried to establish a relationship between the *Toxoplasma* genotype and the severity of clinical manifestations, with inconclusive results [33].

With respect to diagnostic confirmation by serological testing, it should be emphasized that, in contrast to non-infected infants, serum anti-*Toxoplasma* IgA and/or IgM antibodies were detected in all infants in whom infection was confirmed. This finding diverges from reports indicating that the sensitivity of such antibody tests is lower when mothers are infected at the beginning of pregnancy [28, 29]. Neonatal screening, therefore, would not detect infants whose mothers were infected during early pregnancy, when the risk of fetal involvement is higher [28], a finding not observed in the present study.

In conclusion, the demonstration of an elevated incidence of CT in the region of Ribeirão Preto, Brazil, based on the analysis of a representative population sample, reveals the need for the institution of preventive measures, considering that 60% of affected infants may not have been identified by routine clinical examination. The main measure would be the implementation of informative campaigns for the general population and health-care professional, to promote the primary prevention of gestational infection. Neonatal screening by serological testing of filter-paper samples is feasible and

may be widely available. However, tests with a better performance are necessary. Positive screening results must be carefully confirmed by serological tests and follow-up, particularly in asymptomatic children.

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