High frequency detection of *Toxoplasma gondii* DNA in human neonatal tissue from Libya

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**Background:** *Toxoplasma gondii* is a parasite that causes significant disease in humans. Toxoplasmosis is normally asymptomatic, unless associated with congenital transmission, or in immunocompromised people. Congenital transmission generally occurs at low frequencies. In this study, we use PCR to investigate possible congenital transmission of *T. gondii* during pregnancy in a cohort of mothers from Libya.

**Methods:** Two hundred and seventy two pregnant women (producing 276 neonates) were recruited to obtain umbilical cord tissue from their neonates at birth; DNA was extracted from that tissue and tested for *T. gondii* DNA using two specific PCR protocols based on the *sag 1* and *sag 3* genes.

**Results:** *Toxoplasma gondii* DNA was detected in the umbilical cord DNA from 27 of the 276 neonates giving a prevalence of 9.9% (95% CI 6.8–13.9%). Compared with more commonly reported rates of congenital transmission of 0.1% of live births, this is high. There was no association of infection with unsuccessful pregnancy.

**Conclusions:** This study shows a high frequency presence of *T. gondii* DNA associated with neonatal tissue at birth in this cohort of 276 neonates from Libya. Although PCR cannot detect living parasites, there is the possibility that this indicates a higher than usual frequency of congenital transmission.

**Keywords:** Congenital infection, Libya, Miscarriage, Neonatal, PCR, *Toxoplasma gondii*

**Introduction**

Toxoplasmosis is caused by the protozoan parasite *Toxoplasma gondii* and is an important human and animal disease with a worldwide distribution.¹ The prevalence of infection in the human population is estimated at 30% globally. However prevalence varies with values of 10–20% recorded in the UK, China and US but exceeds 40% in some countries, for example, within South America or continental Europe.² Human infection is acquired through contact with oocysts passed in faeces by the definitive host, the cat, or by consuming the tissue cyst(s) found in uncooked or undercooked meat. Congenital transmission, from mother to baby, is also considered a route of transmission – although is considered as rare in humans with occurrence in less than 1 in 1000 live births.¹³⁻⁵ Toxoplasma infection is usually asymptomatic. However, in pregnant women a novel infection can cause congenital toxoplasmosis in the foetus, neonate and developing child and some of them may also develop brain diseases such as bipolar disorder and schizophrenia.⁶⁻⁹ Studies on prevalence in pregnant women, for example in China,¹⁰ show that the frequency of infection usually follows the general population prevalence—in this example, averaging 10% in both cases. However, chronic infection of the mother usually does not result in transmission to the foetus and transmission is generally thought to be related to seroconversion during pregnancy⁸ or possibly, under some circumstances, reinfection of a chronic infection.¹¹

In humans, an estimated 10% of prenatally acquired infections result in abortion (i.e., spontaneous miscarriage) or neonatal death.¹²,¹³ Furthermore, an additional 10–23% of infected newborns will show signs and symptoms present at birth such as retinochoroiditis, intracranial calcifications and hydrocephalus.¹²,¹⁴,¹⁵ It is these abnormalities that significantly reduce...
the quality of life of children that survive prenatal infection of the mother.\textsuperscript{3,5}

While studies on congenital transmission in humans suggest that it may occur rarely in the absence of infection during pregnancy, some recent studies in other species have, controversially, proposed that congenital infection may be occurring much more frequently than previously thought. A number of studies, conducted over many years, using experimental infection of rodents has shown that congenital infection can occur frequently.\textsuperscript{16–18} However, the frequency of this mode of transmission in natural populations of animals, or humans, is less clear. PCR based studies on the transmission of \textit{T. gondii} in several mammalian species, e.g., mice, woodmouse\textsuperscript{17,19–21} and sheep\textsuperscript{22–25} have suggested that congenital transmission may be occurring at a higher frequency than previously thought. Although higher frequencies of congenital transmission are reported in these studies, in many cases there seems to be no measurable disease effect on the neonates in these species. This might suggest that congenital infection may result in apparently healthy carriers. There is some debate, however, as to the generality and importance of these results – especially in sheep.\textsuperscript{16–18}

There are some challenges in measuring congenital transmission rates in natural human populations in ways that are not detrimental to the mother or neonate. The standard approach is to measure the seroprevalence using a variety of serological approaches.\textsuperscript{4} However, some studies have suggested that more sensitive techniques, such as PCR or parasite detection by bioassay, are capable of detecting parasites in seronegative individuals (e.g., in rodents,\textsuperscript{29,30} pigs\textsuperscript{31} and humans\textsuperscript{32}). In the PCR based studies on sheep,\textsuperscript{22,23} the measurement of congenital transmission was conducted utilising umbilical cord tissue as a proxy for neonatal tissue. In those studies, comparison of PCR detection of \textit{Toxoplasma} DNA in umbilical cord tissue with internal tissues (brain) from infected and uninfected aborted lambs showed very strong agreement. These results indicated that PCR positive umbilical cord tissue was a very good predictor for infection of the brain. This offers the possibility of using a similar approach to investigate congenital transmission in humans.

Little is known about congenital or general \textit{T. gondii} infection in humans in Libya. A study of 4280 people from Tripoli was conducted in 1987 and showed prevalence of 51.6\% (1032/2000) in adult males, 43.3\% (130/300) in adult females and 43.7\% (865/1980) in schoolchildren.\textsuperscript{33} While another study, conducted specifically on pregnant women in Benghazi (n=369), found that 47.4\% (175/369) were infected with \textit{T. gondii}.\textsuperscript{34} To our knowledge, no studies have been conducted on congenital transmission in humans in Libya. The objectives of this study were, first, to use PCR detection of \textit{T. gondii} DNA as a tool to investigate the presence of \textit{T. gondii} in umbilical cord tissue at birth to gain an assessment of possible congenital transmission and, secondly, to investigate the relationship between presence of \textit{T. gondii} DNA and clinical or demographic history.

Materials and methods

Sample collection and ethical approval

A total of 272 mothers were recruited during antenatal consultations at Misurata Central Hospital, Libya, in the period prior to the Libyan crisis (2011) when civil war broke out. The participating mothers originated from several cities across the West of Libya (Misurata n=235, Zlit n=17, Tawarga n=9, Al Khums n=6, Sirte n=2, Qaminis n=1, Tripoli n=1, unknown location n=1). Ethical procedures followed local Libyan regulations at the Hospital and both informed patient consent and sample collection was overseen by one of the authors (MSE, Consultant Obstetrician) but followed protocols approved for a parallel study in the UK which was awarded ethical approval by the NHS National Research Ethics Service (ref O5/Q1409/105). University of Salford ethical approval was given by the Research Governance and Ethics Committee, RGEC04/16, and the Research Ethics Panel, REP09/102. Informed consent was obtained from mothers during antenatal sessions to allow collection of discarded umbilical cord tissue, allow completion of a questionnaire and allow information to be obtained from the clinical notes regarding the current birth. Both written information (translated into Arabic) and verbal explanations were provided to ensure understanding and subjects were coded to ensure anonymity with respect to samples. Subjects were free to withdraw at any stage in the consent, collection or post collection process. At collection, samples containing 1 cm\textsuperscript{3} of tissue (i.e., umbilical cord) were collected aseptically and washed using sterile saline to remove any maternal fluids. Using sterile scissors, they were sheared into small pieces and placed into tubes containing 400 \textmu l of lysis buffer (0·1 M Tris-HCl [pH 8·0], 0·2 M NaCl, 5 mM EDTA, 0·4\% SDS), 4\textmu l of Proteinase K (50 mg/ml) and the contents were mixed thoroughly using a vortex for a few seconds. The tube was then incubated overnight at 56°C to assist with cell lysis and the crude DNA extract was then stored at −20°C until further purification. Further DNA purification was carried out using phenol/chloroform/isoamyl alcohol as previously described.\textsuperscript{22} Purified DNA was tested by PCR using primers to the mammalian tubulin gene to ensure the suitability of the DNA for PCR as previously described.\textsuperscript{22} Appropriate protocols were used at each stage of the DNA purification and any subsequent PCR amplifications to prevent cross-contamination as described previously.\textsuperscript{21,23,25,35}

PCR detection of \textit{T. gondii}

Detection of \textit{T. gondii} was carried out using a nested PCR amplification of both surface antigen gene 1 (sag 1) as described previously\textsuperscript{24,36} and surface antigen gene 3 (sag 3)\textsuperscript{37} with subsequent modifications.\textsuperscript{38} Pure \textit{T. gondii} DNA from RH, Martin, 17695 and COR strains were used as positive controls in all PCR experiments. All samples were tested a minimum of three times with a PCR to detect the sag 1 gene and considered positive when three successful PCR amplifications were achieved. Occasional or sporadic positive reactions were retested and considered negative if they could not be amplified to satisfy the three successful amplification criteria. Samples found to be positive using the sag 1 PCR criteria were also confirmed using sag 3 PCR which amplifies from an independent gene locus. Samples negative for sag 1 PCR were also confirmed as negative by sag 3 PCR.

Statistical analysis

Statistical associations were carried out using 2×2 contingency tables, with Fisher’s exact test. Correlation tests involving classes
of data (e.g., age and weight classes) were carried out using a
rank correlation test (Spearman’s). Comparisons of observed with
expected data were carried out using χ² analysis on Microsoft
Excel® spreadsheet software (Microsoft Corp., Redmond, WA,
USA). A p-value of less than 0.05 was used to indicate statistical
significance.

Results

A total of 272 Libyan mothers consented to participate in this
study to investigate the frequency of detection of T. gondii DNA
in foetal-derived umbilical cord tissue at birth. Maternal age
ranges, at the time of the current birth, varied from 17 to 45
years, with a median age of 27 years (IQR 24–32 years). This
cohort of mothers gave birth to 276 babies (268 singletons
and eight twins). Of the newborns, 51.1% (141/276) were female and 47.8% (132/276) were male, and 1.1% (3/276) had unre-
corded gender in the available clinical records. Furthermore,
furthermore, five recorded in this study were
and eight twins). Of the newborns, 51.1% (141/276) were female and 47.8% (132/276) were male, and 1.1% (3/276) had unre-
corded gender in the available clinical records. Furthermore, five
miscarriages (1.8%) were unsuccessful resulting in intrauterine
foetal death of the neonate (all singletons in this instance: three
male and two females). The subsequent determination of cause
of death of these neonates was not recorded in the clinical
notes made available to the study team. Clinical records were
used to determine historical childbirth data, including live and
miscarried babies for each mother. Historical data from only
271 mothers could be obtained. This cohort of mothers had a
variably pregnant (including miscarried births) ranging
between 1 and 15 (median, 3; IQR 1–5; n=271) births per
mother. Sixty of the mothers had had one or more miscarriages
(including the five recorded from the current cohort of births)
totalling the loss of 90 potential neonates. The number of mis-
carriages per mother ranged from 0 (n=211) to between one
and five (median, 1; IQR 1–2; n=60). Four of the five current mis-
carriages recorded in this study were first time miscarriages
while the other mother had also miscarried once previously, but
had previously also had six successful births.

The risk of miscarriage with maternal age is presented in
Table 1. The rate of miscarriage in current (1.8%, 5/272) and
previous pregnancies was high with 21.7% (60/272) mothers
experiencing miscarriage at some point. A strong positive cor-
rrelation between the age class of the mothers and the propor-
tions of mothers with miscarriages was observed (correlation
co-efficient=0.88, n=7, p<0.05).

T. gondii DNA was detected in 27 of the 276 foetal-derived
umbilical cord samples (Table 2), representing the 272 pregnan-
cies. This gave a detection prevalence of 9.9% (27/272) of preg-
nancies (95% CI 6.8–13.9%). Of the successful pregnancies,
resulting in the birth of live babies, 10.1% (27/267) of foetal-
derived umbilical cord tissue showed positive detection of
T. gondii DNA. T. gondii DNA was not detected in the umbilical
cord of any of the five miscarried neonates. Therefore, no associ-
ation was found between presence of T. gondii DNA and unsuc-
cessful pregnancies (p=1.00). Of 273 newborns with available
gender information, 9.2% (13/141) males and 10.6% (14/132)
females were Toxoplasma positive and there was no significant
effect of gender on T. gondii DNA presence (p=0.84). To investi-
gate whether a history of miscarriage was associated with pres-
ence of T. gondii DNA in the foetal umbilical cord tissue, clinical
records were examined. Of the 27 tissue samples positive for
T. gondii DNA, six were neonates from mothers that had suf-
fered at least one historical miscarriage. There was no associ-
ation between history of previous miscarriage and T. gondii
positivity (p=0.81) nor total miscarriage history (i.e., including
the current miscarriage) (p=1.00). The birthweight of the cohort
of babies ranged from 1.3 kg to 5 kg. To investigate whether there
was any association between T. gondii positivity and weight of
baby at birth, newborns were categorised into weight classes,
and there was no significant difference (χ²=10.4; df=6; p=0.89)
in frequency of T. gondii positivity in difference weight classes.

<table>
<thead>
<tr>
<th>Age Class (years)</th>
<th>Total Mothers</th>
<th>Current Pregnancy</th>
<th>Previous Pregnancies</th>
<th>All Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Mothers</td>
<td>Number of Mothers with miscarriage</td>
<td>% of miscarriage</td>
<td>Number of Mothers with miscarriage</td>
</tr>
<tr>
<td>17–20</td>
<td>23</td>
<td>0</td>
<td>0.00</td>
<td>2</td>
</tr>
<tr>
<td>21–24</td>
<td>68</td>
<td>1</td>
<td>1.50</td>
<td>4</td>
</tr>
<tr>
<td>25–28</td>
<td>60</td>
<td>0</td>
<td>0.00</td>
<td>13</td>
</tr>
<tr>
<td>29–32</td>
<td>57</td>
<td>2</td>
<td>3.50</td>
<td>13</td>
</tr>
<tr>
<td>33–36</td>
<td>34</td>
<td>0</td>
<td>0.00</td>
<td>8</td>
</tr>
<tr>
<td>37–40</td>
<td>25</td>
<td>1</td>
<td>4.00</td>
<td>11</td>
</tr>
<tr>
<td>≥41</td>
<td>4</td>
<td>1</td>
<td>25.00</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>271</td>
<td>5</td>
<td>1.80</td>
<td>54</td>
</tr>
</tbody>
</table>

Columns refer to the current pregnancy (those included in this study), previous pregnancies (covering historical miscarriage data) and all preg-
nancies (current and previous miscarriages combined).

One mother had no data.
Discussion

The results of the current study indicated that high frequencies of *T. gondii* DNA could be detected in the foetal-derived umbilical cord tissue samples of neonates born in Libya. One could hypothesise that there is a risk of congenital transmission of the parasite *T. gondii* occurring from mother to baby. If this is the case, then there is a significant implication. As the transmission rate is significantly higher than previously published estimates (less than 1 in 1000 live births1,3–5) the implication is that if parasite transmission is occurring, there are potentially a considerable number of infected babies being born apparently asymptptomatically (at least at birth). For example in this study, of 27 *T. gondii* PCR positive umbilical cord samples from the newborns, all were born without miscarriage and without any observable post-natal defects typically associated with *T. gondii* (e.g., hydrocephalus). In fact, some studies have evidenced that, in general, 80–90% of congenitally infected neonates show no clinical symptoms at birth.39,40 Although none of the neonates in this study showed any detectable symptoms (100%, 27/27), with the relatively small sample size of *T. gondii* positive neonates, this is not significantly different from 80–90% found in the previously reported studies.39,40

One of the limitations of this study is that detection is based on PCR and this means that it is possible that it does not represent living parasites, but parasite DNA molecules. While this has to be acknowledged, PCR is used widely as a diagnostic tool for *Toxoplasma*1 and a wide range of other protozoan parasitic diseases.41 The presence of parasite DNA does, however, indicate the presence, and proximity, of the parasite to the foetus during these pregnancies. It is, perhaps, surprising that none of the occurrences of miscarriage, seen in this study, appeared to be associated with the presence of *T. gondii* DNA. In this cohort of mothers, the current and historic levels of miscarriage were high.

An important contributing factor to newborn mortality has often been thought to be the activity of parasites and infectious agents. *Toxoplasma* is well known as an agent that causes miscarriage.5 In this study there was no relationship between infection and miscarriage. However, the sample size of miscarried neonates was small (n=5). In the case of unsuccessful pregnancies, none of the samples showed the presence of the parasite DNA in the foetal umbilical cord samples. Furthermore, *T. gondii* was detected at a higher frequency from the umbilical cord samples of successful pregnancies (10.1%, 27/267) than would be expected from previously published estimates on congenital transmission rates of around 0.1%. This raises the question as to whether live born babies might be symptomatic or asymptomatic carriers of the infection. In this study it was not possible to follow up individuals – this is an important aspect that could be considered in future studies since congenital infection is often associated with late onset clinical entities such as ocular toxoplasmosis. However, if congenitally infected children were frequently asymptomatic, and infection persisted through adulthood, it might offer the opportunity for the parasite to be passed down vertically or via serial congenital transmission in human populations.26

Few studies have been conducted on vertical transmission in humans but some evidence for this may be accumulated from animal species. Watson and Beverley42 studied sheep *Toxoplasma* and showed that infection of the placenta corresponded to infection of sacrificed live born lambs, thus demonstrating a link between maternal and foetal infection. Further recent studies on sheep have revealed that congenital transmission may be sufficient to explain the maintenance of *T. gondii* in natural populations of sheep without requiring new infections by oocysts secreted by cats.22,23 In the case of these sheep studies, it was demonstrated that PCR detection of *T. gondii* DNA in the umbilical cord was a good indicator of infection when aborted lamb brain tissue was compared in the same animal. Nevertheless, a challenge to be encountered in humans (as may be the case in sheep and other animals) is to understand whether these individuals are truly asymptomatic carriers of vertical transmission. In the case of the sheep studies24,25 detailed family pedigrees were established which demonstrated the occurrence of familial transmission. Further work remains to be done to determine the role of vertical transmission of *Toxoplasma* in humans.

Only a few studies exist on *T. gondii* prevalence in Libya and they demonstrate high prevalences (40–50%)33,34 compared to many other areas of the world. Although updates on these baseline studies have not been done, the relatively high levels of detection of *T. gondii* in the foetal-derived tissues, reported in this study, are consistent with these high prevalence levels. Similar high prevalence levels of *T. gondii* have been reported in pregnant women from continental Europe: Spain 28.6%, Sweden 18%, Belgium 48%, Netherlands 35%, Greece 36% and Germany 63%.2 However, in these other studies only 0.2% congenital transmission was shown to be occurring,43 based on proven clinical cases of infection. In comparison the reported rate of 9.9% (27/272) reported in this study, based on PCR, is very high. The difference can be explained by differences in the detection and diagnostic systems used but interestingly may suggest higher levels of transmission of parasites than appear as clinically proven cases.

Unlike the current study, previous reports have generally used serology for the diagnosis of *T. gondii* in human tissues. Serology is the classical diagnostic modality consisting of several tests on which, virtually all *Toxoplasma* prevalence studies are carried out44 but a variety of different approaches are used. Most of these require the determination of antibody cut off prior

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**Table 2.** PCR detection of *Toxoplasma gondii* DNA in successful and unsuccessful pregnancies from a collection of 272 mothers

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Successful pregnancies</th>
<th>Unsuccessful pregnancies</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of pregnancies</td>
<td>267</td>
<td>5</td>
<td>272</td>
</tr>
<tr>
<td>Number of PCR positives</td>
<td>27</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>Total %</td>
<td>10.11</td>
<td>0</td>
<td>9.93</td>
</tr>
</tbody>
</table>

A successful pregnancy was defined as one in which all neonates were born alive. An unsuccessful pregnancy was described as a pregnancy in which one or more babies suffered intrauterine foetal death. In the cases of mothers (n=4) with twin neonates (n=8), all newborns were *T. gondii* DNA negative.
to use and there is some variation in standardisation globally. The gold standard for testing for *T. gondii* is serology which has been used by experts in the field to now routinely record the serological method used and antibody cut-offs when reporting prevalence studies. This enables a judgement of the relative validity of results. In the current study only molecular methods were used to detect for congenital transmission in babies. A possibility exists that, if congenital transmission is being detected in this study, differences in the approaches to detection of the parasite may account for these unusually high transmission frequencies. In sheep, Mason et al., demonstrated that seronegative neonatal lambs could be PCR positive and the two approaches (serology vs PCR) showed poor agreement when comparisons were made. This is suggestive that the differences in measurements of congenital transmission could be due to differences in the methodology of measurement. Further work may still be required to establish robust protocols for investigating congenital transmission frequency.

Clinical cases of congenital infection have been observed to occur from chronically infected mothers in some rare instances. These may either be caused by reactivation of cysts or reinfection. It has been suggested that the natural state of immunosuppression entered into by the mother during pregnancy could be hijacked by the parasite. In this case it could use the opportunity to reactivate from tissue cysts, as tachyzoites, which then cross the placenta to infect the foetus. In this case it could use the opportunity to reactivate from tissue cysts, as tachyzoites, which then cross the placenta to infect the foetus. Although most studies report little evidence for reactivation, some studies suggest this might be a possibility. Further work would be required to investigate this question.

There are a number of shortcomings of this study which we recognise have an influence on the strength with which the results can be interpreted. Firstly, while data on historical and current miscarriages were collected from records, serological data on screening/diagnosis of mothers for *T. gondii* infection was not conducted. It was therefore, not possible to determine whether mothers had a chronic infection or sero-converted during pregnancy. In the case of those babies with detectable *T. gondii* DNA, it is therefore not possible to determine how or at what stage the mothers became infected. It is not possible, therefore, to address chronic reactivation versus infection during pregnancy with this dataset. Furthermore, it was not possible to get access to blood from the babies and, therefore, not able to confirm diagnosis by serology. As discussed previously, based on PCR evidence alone it is not formally possible to conclude that the babies had active infections. Ideally, future studies should involve early recruitment of mothers and monitoring of infection throughout pregnancy by collection of serum samples. Suitable neonatal samples (e.g., cord blood) for *T. gondii* testing would need to be collected to try to link the PCR positivity with infection in the newborns. Finally, a long term follow up study of the newborns could provide important clinical data with respect to the consequences of infection. Unfortunately, at the time of writing the political difficulties in Libya are likely to make such future studies challenging.

Conclusions

This study shows a high frequency of detection of *T. gondii* DNA in umbilical tissue from neonates in Libya although in this study there was no association with miscarriage. While we recognise that there are caveats associated with our interpretations, it raises the possibility that the foetus could be potentially exposed to *T. gondii* infection with higher frequency than previous studies suggested. It also raises questions about the methods used to detect transmission to the foetus, the importance and mechanism of congenital transmission and the potential generation of asymptomatic carriers of vertical transmission in humans. Future studies are required to explore these important questions further.

Authors' contributions: SZHH, MSA, OG, DT and GH conceived the study. MSE conducted the clinical assessment of patients in Libya. MSA, OG and MSE conducted sample collection in Libya. DT, MSE and GH considered ethical issues and MSE implemented local ethical processes in Libya. Laboratory work was conducted by MSA, OG, JMH, EAW and DT. Data analysis and interpretation was conducted by SZHH, MSA, OG, ZRL and GH. SZHH and GH drafted the manuscript but all authors contributed to the writing of it. SZHH, ZRL and GH critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. GH is the guarantor of the paper.

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Competing interests: None declared.

Ethical approval: Ethical procedures followed local Libyan regulations at the Misurata Hospital. University of Salford ethical approval was given by the Research Governance and Ethics Committee, RGEC04/16, and the Research Ethics Panel, REP09/102.

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