

1 **A review of performance of Zika assays in the context of TORCH diagnostics**

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17 **Running title: TORCH and perinatal Zika diagnostic challenges**

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### 53 **SUMMARY**

54 Infections during pregnancy that may cause congenital abnormalities have been recognized  
55 for decades, but their diagnosis is notoriously challenging. This was recently again illustrated  
56 with the emergence of Zika virus (ZIKV), highlighting the inherent difficulties in estimating  
57 the extent of pre- and postnatal ZIKV complications, because of the difficulties in establishing  
58 definitive diagnoses. We reviewed the epidemiology, infection kinetics and diagnostic  
59 methods used in *Toxoplasma gondii*, Parvovirus B19, Rubella virus and Cytomegalovirus  
60 (TORCH) infections and compared that with current knowledge of ZIKV diagnostics to  
61 provide a basis for the inclusion of ZIKV in the TORCH complex evaluations.

62 Similarities between TORCH pathogens and ZIKV support inclusion of ZIKV as an emerging  
63 TORCH infection. Our review evaluates diagnostic performance for maternal screening, fetal  
64 screening and neonatal screening. We show that sensitivity, specificity, and positive and  
65 negative predictive value of TORCH complex pathogens are wide widely variable, stressing  
66 the importance of confirmatory testing and the need for novel techniques for earlier and more  
67 accurate diagnosis of maternal and congenital infections. In this context it is also important to  
68 acknowledge different needs and access to care for different geographic and resource settings.

69

### 70 **INTRODUCTION**

71 The present Zika virus (ZIKV) epidemic was first noted through an alert from the Brazilian  
72 health authorities notifying the World Health Organization (WHO) of an illness characterized  
73 by skin rash in north-eastern states and subsequently signalling an almost 20-fold increase in

74 the incidence of microcephaly in newborns coinciding with rapid spread of ZIKV after  
75 incursion into the continent [1,2,3,4]. Until then, ZIKV infection was generally assumed to be  
76 associated with mild and transient disease, estimated to be asymptomatic in approximately  
77 80% of the cases [5]. Hence, it is likely that the infection is underdiagnosed or underreported  
78 in a disease-endemic setting [6].

79 The association of ZIKV infection with congenital neurological disease has since then been  
80 subject of numerous publications. First establishment of ZIKV causal association with  
81 neuropathological processes came from a study showing widespread ZIKV infection in the  
82 brain of a fetus from a pregnancy that had been terminated due to severe fetal malformation  
83 [7]. Further evidence for the association came from larger case series, retrospective analysis  
84 of notification data from regions with prior outbreaks [8], and replication of the syndrome in  
85 animal models [9,10,11,12,13]. Although there is general agreement of such association,  
86 many uncertainties remain with regard to actual risk of fetal infection during pregnancy [14].  
87 A systematic review estimated the prevalence of microcephaly at 2.3% (95% CI: 1.0-5.3%  
88 [15], but estimates range widely and little is known about the risk of complications in relation  
89 to timing of maternal infection (first trimester versus later exposures), prior (flavi virus)  
90 exposure, the rate of transplacental transmission in relation to these factors, the rate of fetal  
91 infection, and the rate of congenital disease once infected (Figure 1). Two recent reports  
92 suggest a decreasing risk over the course of pregnancy [16,17]. A recent European study  
93 showed an overall congenital anomaly prevalence of any (non-genetic) cause of 1.5/100 total  
94 births, but for microcephaly interpretation was hampered by differences in diagnostic criteria  
95 [18].

96 At present, ZIKV diagnostic algorithms are based on the use of reverse transcriptase-  
97 polymerase chain reaction (RT-PCR) for virus detection and/or serological determination of  
98 pathogen-specific IgM and IgG antibodies supplemented with virus neutralisation assays if

99 available. All of the assays have benefits but also known limitations, challenging  
100 interpretation at different stages of pregnancy, particularly in relation to the wide diversity of  
101 flavivirus background exposures in the regions where ZIKV circulates. Virus genome  
102 detection by RT-PCR is considered confirmatory but has a very short detection window as  
103 ZIKV viremia is short, although virus may persist for longer periods in other body fluids, with  
104 reported persistence up to 120 days for semen [19]. Also pregnant women may experience  
105 prolonged viremia [20,21], with reported (transient) presence of ZIKV in fetal blood and  
106 amniotic fluid described in a well described small case series [21]. Antibody based testing is  
107 severely hampered by cross-reactivity with antibodies from prior flavivirus exposures.  
108 Other infections during pregnancy are associated with congenital and subsequent neonatal  
109 disease, sometimes referred to as TORCH infections (*Toxoplasma gondii* (TOXO), Other  
110 [e.g. varicella zoster, parvovirus B19 (PB19)], Rubella (RV), Cytomegalovirus (CMV) and  
111 Herpes simplex (HSV) with or without syphilis [22]. Diagnosis of fetal infection and linking  
112 fetal infections to clinical outcomes requires knowledge of infection kinetics, including timing  
113 and differentiation of primary from nonprimary infection (i.e. re-activation, re-infection),  
114 maternal and fetal immune response in relation to pathology, and availability of biomarkers  
115 predictive of vertical transmission and presence and/or severity of fetal abnormalities  
116 [23,24,25]. For instance, TOXO and CMV cause persistent or latent infections, whereas RV,  
117 PB19 and ZIKV infection are thought to be primarily self-limiting. Immunocompetent  
118 pregnant women with previous infection with TOXO are considered not at risk for congenital  
119 abnormalities, whereas for CMV primary as well as subsequent infections are associated with  
120 congenital infection and abnormalities, albeit with a lower attributable risk. The recent ZIKV  
121 epidemic and its possible association with microcephaly has initiated the discussion to include  
122 ZIKV as novel TORCH pathogen [26].

123 Although the described maternal infections are an important cause of fetal and neonatal  
124 morbidity and mortality, on a global scale, the overall contribution to fetal and congenital  
125 diseases is limited [27,28,29] due to cumulative low and/or limited a priori risks of maternal  
126 infection, vertical transmission and subsequent congenital infection (Figure 1).

127 Generally, risk of congenital disease following maternal infection is linked to primary  
128 infection and timing in pregnancy infection, which is pathogen dependent  
129 [25,30,31,32,33,34,35,36,37]. Non-primary maternal infections may result in fetal  
130 transmission, but only in the case of CMV does this contribute significantly to congenital  
131 disease [38].

132 The low attributive risk of TORCH and ZIKV infections to congenital disease has  
133 consequences for diagnostic accuracy and the ability to provide information relevant for  
134 clinical decision-making. This is further complicated by the high proportion of asymptomatic  
135 maternal infections [6,35,39,40,41], challenging timely detection of fetal infection and early  
136 neonatal congenital disease, which may remain asymptomatic for years [40,42]. Early  
137 diagnosis of fetal disease risk in pregnancy, however, is important particularly when early  
138 therapeutic management is available eg. in TOXO [43]. Consequently, diagnostic algorithms  
139 should reliably and timely detect maternal infection, determine (risk of) vertical transmission  
140 and establish or exclude congenital infection. This review assesses diagnostic methods  
141 presently used for TORCH infections, their correlation with congenital and/or neonatal  
142 disease, their predictive value in prenatal screening; it will document gaps in methods used;  
143 and it will draw implications for diagnostic algorithms development in novel or (re)emerging  
144 infections such as Zika virus.

145

146 **EPIDEMIOLOGY, INFECTION AND TRANSMISSION RISKS OF SELECTED**  
147 **TORCH PATHOGENS (FIGURE 1)**

148 The risk of infection during pregnancy varies by pathogen and depends upon geographic  
149 region, prevalence in the population, and preventative (vaccination) practices.

150 The seroprevalence of *Toxoplasma gondii* among women of childbearing age shows a broad  
151 range, from <2% in a large Chinese cohort [44] up to 75% in Brazil [45], with a mean  
152 estimate of around 40% [46,47,48]. Similar broad ranges in seroprevalence between 30-72%  
153 are reported for PB19 and CMV [49,50,51,52,53,54,55,56] although for CMV  
154 seroprevalences up to 100% are also reported [57]. RV seroprevalence estimates depend on  
155 (differences in) vaccination practices [58,59,60]. ZIKV seroprevalence has a geographic  
156 distribution, varying from <2% in travellers returning from endemic areas or blood donors in  
157 non-endemic settings [61,62,63] to up to 39% in healthy individuals living in endemic areas  
158 [64,65] increasing to >60% following outbreaks [66, 67]. These wide ranges in background  
159 seroprevalence affect the likelihood of primary infection during pregnancy, as well as the  
160 interpretation of diagnostic assays, and need to be taken into account when developing  
161 diagnostic algorithms.

162

163 To assess clinical impact of exposure to TORCH pathogens and ZIKA during pregnancy, it is  
164 important to consider maternal infection risk, fetal transmission risk, and congenital infection  
165 risk for each pathogen (Figure 1). Maternal infection risk (MIR) estimates defined as the  
166 annual infection rate for selected TORCH infections, range from 0.1-0.6% for TOXO [31] to  
167 2-7% for PB19 and CMV [68,69,70,71,72,73], with epidemic rise up to 10% in PB19.  
168 Reliable data for RV MIR is lacking in an elimination setting, but annual incidence is  
169 estimated at 1.3/100.000 pregnancies in the general population [74]. A 6.4% IgG  
170 seroconversion was reported in women with non-immune RV titers prior to pregnancy [75].

171 Although efforts have been made to calculate the maternal infection risk for ZIKV, reliable  
172 data are still lacking, due to several factors including diagnostic limitations (e.g. cross-  
173 reactivity) [76,77], and difference and rapid changes in epidemiology.

174 Fetal transmission risk (FTR), defined as the proportion transplacental transmission following  
175 (primary) infection during pregnancy, is also pathogen dependent and is linked to timing of  
176 infection during pregnancy. FTR may increase (TOXO, PB19, CMV) or decrease (RV)  
177 during the pregnancy period, with a variable mean FTR estimated to range from 24-80% for  
178 these pathogens [25,33,42,78,79]. For ZIKV, FTR is thought to be highest during first  
179 trimester, but more data is needed [16,17]. Perinatal transmission has also been reported for  
180 ZIKV [80].

181 Despite high FTR, the congenital infection risk (CIR) defined as number of congenital  
182 infections per 1000 live births (or number of fetal deaths/hydrops fetalis per 1000 infected  
183 fetuses in PB19) is low, ranging from <1/100000 pregnancies (RV) up to 0,1-20/1000  
184 pregnancies in TOXO, PB19 and CMV [33,34,81,82,83,84,85,86,87,88,89]. Overall  
185 congenital CMV infection is most prevalent in the developed world (5-20/1000 live births)  
186 [30,90], followed by toxoplasmosis (0.1-5/1000 live births) [31,46], and RV (annual  
187 incidence 0.4/100.000)[81]. Parvovirus B19 is associated with hydrops fetalis [82,83,84] and  
188 fetal death, with an estimated annual incidence of <4/1000 fetuses [85]. Recent studies  
189 estimated the contribution of symptomatic ZIKV during pregnancy to ZIKV associated  
190 congenital disease at 7% [17], and evidence of acute infection in pregnancy at less than 4%  
191 [77]. How this translates to the overall contribution of ZIKV to e.g. congenital microcephaly  
192 prevalence depends on the baseline risk and these are uncertain [18,91]. A retrospective  
193 analysis in French Polynesia estimated a risk of microcephaly associated with ZIKV infection  
194 at 9.5/1000 pregnancies, with an overall risk of microcephaly in 0.2/1000 neonates [8].



195 CIR does not necessarily follow the FTR, with highest CIR in the 1<sup>st</sup> trimester for TOXO, RV  
196 and CMV [25,35,34,42] and highest CIR in the second trimester for PB19 [36,37]. Also, for  
197 ZIKV highest risk is reported in 1<sup>st</sup> trimester [8,17]. Overall CIR is limited to primary  
198 infection, except for CMV, where re-infection or re-activation contributes mainly to  
199 congenital CMV disease burden [56,92]. Latent or chronic TOXO infection does not exclude  
200 transmission but does not result in CIR in non-immunocompromised pregnancies [93,94]. In  
201 general, the low attributive risk of the reported infections to overall prevalence of congenital  
202 disease [27,28,29] impacts on the performance of diagnostic assays.  
203 This implies that the low a priori attributive risk of TORCH and ZIKV to congenital  
204 infections needs to be included in every step excluding or confirming maternal, fetal and/or  
205 congenital infection.

206

## 207 **MATERNAL DIAGNOSTIC TESTING (FIGURES 2A – 2D)**

### 208 **Infection kinetics**

209 Interpretation of diagnostic testing during pregnancy requires knowledge on infection kinetics  
210 defined by prevalence and duration of symptoms, duration of pathogen presence in different  
211 body fluids, loads, timing of development of specific antibodies, background antibody  
212 prevalence, and relationship between these parameters. Molecular detection in early  
213 symptomatic infection is generally considered proof of acute primary infection [17,40,59,95],  
214 although not true for each pathogen, i.e. CMV. Primary infections typically show IgM and  
215 IgG development, determined with serological assays with or without confirmatory testing  
216 [96,97]. Reported antibody kinetics differ between selected pathogens. TOXO IgM  
217 seroconversion occurs relatively late between 15-30 days [98,99], whilst early IgM rise is  
218 observed for PB19 towards the end of the first week of infection coinciding with peak viremia  
219 [56] as well as for RV whose IgM rise within 5 days after rash onset [100]. CMV IgM may

220 become detectable between 0-3 weeks [101] with peak IgM observed between 1-3 months  
221 [102]. IgM antibodies against ZIKV also show an early rise and can be first detected within  
222 the first week after clinical symptoms, but also IgG antibodies can be detected within the first  
223 two weeks [103].

224 PB19 IgM can persist up to 3 months postinfection [104], and ZIKV IgM can also persist  
225 beyond 3 months with a reported wide range [105]. In addition, long-term persistence of  
226 rubella IgM is reported following vaccination [106,107,108], due to natural occurrence of  
227 non-specific IgM [109,110] and despite attempts to improve assays [111,112,113,114].  
228 Differentiation of acute infection from latent infection or re-activation / re-infection is  
229 important in TOXO and CMV, as IgM/IgG may coincide thereby making it difficult to  
230 diagnose primary infection if first consultation yields an IgM/IgG positive test result [115]. In  
231 this case, confirmatory testing is needed, e.g., by AI or IB with presently available assays.  
232 [47,116,117]. Development of CMV specific IgG with a negative sample collected earlier in  
233 pregnancy is considered proof of primary CMV infection, although in absence of routine  
234 screening this is usually not feasible [118].

235

### 236 **Molecular assay performance and limitations**

237 Although PCR assay specificity is high in acute primary infection, the window of PCR  
238 positivity may be short as shown for PB19 [95] [Figure 2]. Limited data from ZIKV showed  
239 a similar pattern. A recent external quality assessment (EQA) suggested similar more robust  
240 specificity and variable sensitivity between labs [119]. Furthermore, most acute (primary)  
241 infections in pregnancy are asymptomatic, and day of infection is unknown precluding use of  
242 this gold standard test. In primary PB19 in pregnancy, high viral load in acute infection is  
243 associated with early positive IgM [39,56,120,121]. Long-term, low load, DNA (desoxyribo  
244 nucleic acid) persistence is observed following PB19 infection [104,122]. In one study, the

245 use of endonuclease treatment before molecular testing differentiated naked DNA persistence  
246 from true viremia [123]. For ZIKV, rapid degradation of RNA (ribo nucleic acid) was  
247 reported in urine samples [124]. In acute maternal PB19 infection, positive predictive value  
248 (PPV) of PB19 PCR is high, but at time of fetal symptoms, the PPV of PB19 DNA detection  
249 in maternal blood is generally low, as clinical symptoms in the fetus are usually observed when  
250 maternal viremia has ceased. This timely relation is not established for other primary  
251 infections such as TOXO [125,126] or CMV [127]. Viremia in pregnant women is associated  
252 with vertical transmission risk and increased CIR but the relationship between maternal  
253 infection, FTR and CIR is different for different pathogens [128,129,130,131,132,133,134].  
254 Currently, there is no obvious predictor for transmission risk. For instance, viral load does not  
255 differentiate transmitters from non-transmitters in CMV [129,130,135]. Absence of  
256 relationship with maternal disease severity or viral load was also recently described for  
257 congenital ZIKV [136]. Low viral load positives may occasionally not show IgM  
258 seroconversion (RV, ZIKV) [137,138,139], low assay sensitivity was suggested as one of the  
259 possible explanations [139]. Genotype differences may impact sensitivity of assays  
260 [140,141,142] which is important when considering using assays in different regions.

261

## 262 **Serological assays and performance**

### 263 **Primary diagnostic assays**

264 Generally, TORCH immunoassays report relatively high specificity for IgM and IgG or in  
265 IgM negative samples [34,108,113,143] but as the *a priori* likelihood of a maternal infection  
266 with TORCH pathogens generally is low, even a relatively low false positivity rate translates  
267 to a low PPV for all pathogens (including ZIKV) except PB19, stressing the need for  
268 confirmatory testing [144,145,146,147] [figure 2]. Comparative studies of assays, reporting  
269 relative performance data overestimate sensitivity and specificity [108,113, 143, 148]. In view

270 of the above, a positive IgM test result always requires confirmation with other assays  
271 [36,101,113,149] and follow up samples. More specific (recombinant) peptide specific IgM  
272 assays may provide solutions, but their performance also needs to be fully evaluated  
273 [121,150].

274

### 275 **Confirmatory testing**

276 The use of confirmatory testing with avidity index measurements (AI), immunoblots (IB) and  
277 virus neutralisation testing (VNT) is not consistent between pathogens and also show variable  
278 performances (Figure 2). Testing for AI is common practice for TOXO and CMV  
279 diagnostics, but not for PB19 [121] or RV [151]. The rationale for avidity testing is that  
280 avidity of antibodies increases with time, and high AI correlates with infection in the more  
281 distant past [152,153,154,155,156,157,158]. Confirmatory sensitivity of AI depends upon the  
282 initial screening platform used, as shown in one study. A negative initial IgM screening is  
283 unlikely to be confirmed [145]. IgM positivity combined with low AI increases sensitivity and  
284 PPV of the combined assays in diagnosing recent infection [159,160,161,162]. In contrast  
285 (persistent) low AI with positive IgG has a relatively high negative predictive value (NPV)  
286 [163,164]. High AI plus IgG usually confirms past infection, however, for TOXO AI  
287 maturation may never occur [165]. Rapid increase in AI in CMV was associated with false  
288 exclusion of recent infection [166] with higher FTR [167] and CIR [168]. Therefore,  
289 exclusion of acute infection based on (high) AI requires a predefined time window  
290 [101,114,169,170,171], and size of the window varies depending on pathogen, thresholds and  
291 platforms [172]. Different antigens, including recombinant antigens as target for antibody  
292 response may improve AI assay performance [170].

293 Antibody test results may also be confirmed by IB [101,173] or different complementary  
294 assays [174]. Epitope-specific IgG IB used in TOXO and PB19 could confirm IgM/IgG

295 measurements [175,176] particularly in equivocal outcomes [177] or in (false) negative  
296 results with high viral load [146] and helped timing of infection by correlation of IB with  
297 virusneutralisation in CMV [178] or with AI in TOXO [179].

298 An interesting application is the use of IB in TOXO for discrimination of maternal and  
299 neonatal antibody responses by comparing patterns of antibody binding to different proteins  
300 or peptides in blood from mother and neonate [180]. Limitations of IB include lack of  
301 standardisation with variable concordance between assays, particularly in acute infection  
302 [181,182] and different diagnostic accuracy of band patterns in the blots [175]. Predictive  
303 value of IB depends upon the target, and IgM blots often have poorer predictive value  
304 compared to IgG blots [173,183]. Virusneutralisation data are primarily available for CMV  
305 [96], but also commonly used in confirmation of ZIKV infection [184], with generally high  
306 assay performance.

307

### 308 **Limitations of serodiagnostic assays**

309 A major limitation for all diagnostic methods described is interassay variability [98,185], use  
310 of different cut-offs, differences in classifications of positives [185,186], low agreement  
311 between AI index assays [187], variability between platforms  
312 [165,114,171,172,188,189,190,191] and lack of standardization. Another serious concern is  
313 the “grey zone” classification, i.e. the area between the negative outcome and the positive  
314 outcome of a test [98,114,153,163,164,166,186,187,192,193], which differs considerably  
315 between assays [194] and the lack of standardisation of cut-off values for the same assay  
316 [183]. Assigning the grey zone to the negative or positive group impacts on sensitivity or  
317 specificity (as indicated in figure 2, where for example category Tox+ denotes the assignment  
318 of grey zone to the seropositive group and Tox – assignment to the seronegative group)  
319 [148,195,196] stressing the need for common standards for assay development and validation

320 [197,198,199,200,201], use of a standardcurve [202] and/or (international) standardization as  
321 shown in RV [186,203]. Even when general standards for defining seropositivity are applied,  
322 different assays show different performance characteristics which is impacted by the  
323 assignment of equivocal results to the positive or negative outcome [108,204,205]. Since this  
324 mainly affects sensitivity it increases NPV, particularly when prevalence decreases  
325 [92,168,206,207,208].

326

### 327 **FETAL INFECTION DIAGNOSTIC TESTING (FIGURES 3A – 3D)**

328 In case of suspected fetal infection, molecular detection of virus DNA or RNA in amniotic  
329 fluid (AF) or cordblood (CB) is the primary diagnostic option in most cases except in TOXO.  
330 Limited serological data on AF include IgM determination [209,210]. IgG determination is  
331 not informative as it is usually of maternal origin. IgM and/or IgA determination in AF or  
332 fetal blood (FB) have low diagnostic value [211,212,213,214,215], whereas cell culture  
333 isolation (virological confirmation) is more specific for example in CMV [215]. Loads in FB  
334 or AF may be 100-1000-fold higher than in maternal blood [157,129,216], particularly in  
335 symptomatic fetuses [217,218,219] as shown in PB19, CMV and TOXO. Although (viral)  
336 load in primary infection may be high in the fetus [211] its presence is not necessarily  
337 associated with symptomatic infection [220,221,222,223]. Also, normal pregnancy outcome  
338 has been observed in maternal seroconversion without positive AF-PCR [208]. These  
339 discrepancies possibly reflect different windows of infection detection. Overall when  
340 available, PCR on AF or FB has good specificity and NPV in the fetus (Figure 3)  
341 [210,224,225,226,227,228,229] . For PB19, sensitivity has been shown to increase in  
342 presence of maternal viremia [216], and for TOXO with a shorter interval to AF or FB  
343 sampling [224, 226] or use of multicopy genes [230]. Assay performance may be different  
344 between AF and FB, with reported concordance between 73% and 99% [210,213]. Although

345 (transient) ZIKV was reported in AF and FB in fetuses of women with proven infection  
346 during pregnancy [21], there are no quantitative data on FB/AF in Zika available at this point  
347 [231].

348

#### 349 **POSTPARTUM DIAGNOSTIC TESTING (FIGURES 4A – 4D)**

350 Postpartum sequelae of fetal infections have been observed for TOXO, RV, CMV and ZIKV.

351 Although literature is not consistent on this issue, fetal anemia following PB19 infection may  
352 result in severe postpartum sequelae [232]. In general, timely postpartum diagnosis is

353 hampered by low sensitivity of IgM testing [33,36,40,205,228,233,234,235], the presence of

354 maternal antibodies, and the high proportion of asymptomatic CMV or TOXO infected

355 newborns [31,128,224,228,236], unlike for RV [237]. As a consequence, ascertainment of

356 congenital disease typically requires longer-term follow up, posing challenges to the

357 differentiation with postpartum infection [40,238,239].

358 IgM positivity in cord blood or peripheral blood in newborns <24 hours in RV and CMV

359 confirms prenatal infection when supported by viral load testing [233,240]. IgM assay

360 performance is better when testing is done more selectively, in symptomatic neonates as

361 reported for TOXO [218]. Contamination with maternal blood should be excluded within the

362 first 10 days postpartum if first sample was taken from CB, as shown for TOXO[35].

363 The majority of IgG detected at birth will be from maternal origin [31,241], but may be

364 neonatal [242]. Generally maternal IgG is assumed to persist for less than 6 months

365 [243,244], but for example in TOXO persistence of IgG level at 12 months is used to confirm

366 or exclude congenital toxoplasmosis and IgG immunoblot is used to overcome the uncertainty

367 about IgG origin [205,245]. Use of immunoblot or other multi-antigen assays early

368 postpartum [249] has shown to provide an opportunity to differentiate congenital from non-

369 congenital infection by comparing maternal and neonatal antibody binding patterns [246,247].

370 The feasibility of using differences in AI for this purpose has also been studied, e.g. for  
371 TOXO and CMV [247,248,249,250]. Slow IgG AI maturation in neonates, in combination  
372 with IgM correlated with congenital RV [251,252], identical neonatal and maternal AI  
373 excluded congenital toxoplasmosis [35].

374 Molecular testing of neonatal blood or urine has generally good specificity [239,253] with  
375 higher viral load, and longer RNA/DNA persistence in symptomatic babies, particularly in  
376 urine or throat samples for selected pathogens within a selected time frame after birth.  
377 [239,254,255,256]. Viral load has been used to differentiate congenital from postpartum  
378 infection, when early samples are available, but it is not clear if these findings can be  
379 generalized [257].

380

#### 381 **EFFORTS AT IMPROVING DIAGNOSTIC ACCURACY**

382 There have been many efforts to improve diagnostic accuracy, however, this has not yet  
383 resulted in significant improvements. These efforts include development of recombinant  
384 (multi-) proteins and peptide specific tests using different techniques (e.g.,  
385 immunoproteomics) [258] in (multiplex) assays to improve sensitivity and specificity  
386 [143,259,260,261], distinction between primary and postprimary infection [262,263,264],  
387 timing [265], and transmitters [266]. For example, recombinant proteins in novel avidity  
388 assays reported a PPV of >85% [267] and were better suited for IgG detection in TOXO [268]  
389 or could serve as proxies for functional antibody measurements like virus neutralisation in RV  
390 [269]. Multiplex assays are used for simultaneous detection of different antibodies in  
391 TORCH [34,108,113,143] , which is important for differential diagnostic approaches.  
392 However, assays still have the performance limitations of the standard assays described.  
393 Microarray based assays are developed to improve simultaneous testing of antibodies of  
394 different pathogens, including (extended) TORCH [270]. Use of dry blood spots in multiplex



395 serological assays allows use of small volumes, and shorter diagnostic delay [271,272,273],  
396 with potential better assay performance on for example plasmonic gold chip multiplex  
397 immunoassay platforms as shown in TOXO [274,275,276]. Cell mediated immunity (CMI)  
398 assay data (IGRA, ELISpot) particularly come from CMV but is not routinely used. Higher  
399 CD4+/CD8 proliferative T-cell response was associated with primary infection  
400 [277,278,279,280,281], improving assay sensitivity of low IgG avidity [282], but also  
401 reporting different assay performances for example in primary infection and transmitters  
402 [283]. Since CMI in the neonate is never from maternal origin, it is hypothesized that it might  
403 aid in differentiating maternal from foetal ZIKV infection.

404 Rapid point of care testing, such as immunochromatography [98], loop-mediated isothermal  
405 amplification (LAMP) [141,284] or digital microfluidic (DMF) diagnostic platforms [285]  
406 studied for different pathogens, may further reduce time to first positive test, increase  
407 sensitivity and/or decentralised availability in resource limited settings. Such developments  
408 are also reported for ZIKV [286,287, 288,289] Alternative, novel methods in amniotic fluid  
409 samples include comparisons of metabolic profiles (metabolomics) of transmitter vs non-  
410 transmitter infections [290,291], cytokine profiles [292] or peptidome prognostic classifiers  
411 [229] to differentiate infected from non-infected fetuses, or distinguish symptomatic from  
412 asymptomatic infections postpartum. Such developments are particularly important as they  
413 may provide early (prenatal) information on the risk of overt clinical congenital disease  
414 postpartum.. Other non-pathogen related methods are those comparing differential gene or  
415 protein expression between fetal cells and maternal cells [293]. In analogy with previously  
416 developed tests for non-infectious prenatal screening [294,295], genes associated with  
417 neurodevelopment were studied as biomarkers in cell-free RNA transcripts in AF samples  
418 [296]. Paper-based cell-free RNA was recently evaluated for rapid point of care testing of  
419 ZIKV [297].

420

421 **CONCLUSIONS**

422 Our review of approaches to diagnose acute maternal infection, determine vertical  
423 transmission risk and establish presence or absence of congenital infection has shown  
424 similarities but also large variation in approaches between pathogens, risking under-  
425 exploration of methods for optimal diagnostics. Present diagnosis of TORCH and ZIKV  
426 infections is primarily based on serological testing with a focus on IgM and/or IgG detection,  
427 for which a variety of commercial assays is available. These assays show variable  
428 performance and may not differentiate between primary and non-primary infections [115],  
429 persistence or may be limited by cross reactivity [108,112]. A positive serological test thus  
430 always requires confirmatory testing, including IgG avidity index determinations,  
431 immunoblots, virus neutralisation and molecular testing [43,212,224].

432 Use of different assays and lack of (international) standardization hamper the interpretation of  
433 and agreement between different studies [298], despite availability of (WHO) recommended  
434 antigens, primers and probes [299]. Efforts to improve detection of primary infection and  
435 timing in pregnancy have not yet resulted in reliable biomarkers for fetal or congenital disease  
436 risk [300]. Even if protocols and/or algorithms are in place, variability between assays  
437 interferes with unambiguous and timely decision making [301].

438 Thus predefined (more) generic approaches with standardized diagnostic assays and  
439 algorithms are needed to improve adequate and timely diagnosis of (primary) maternal  
440 infections, and subsequent postpartum congenital disease [302], particularly in low endemic  
441 settings where suboptimal diagnostic performance may have an increased risk of false  
442 positive outcomes. Lessons to draw from this review for novel challenges such as ZIKV are to  
443 directly combine methods [52], increase epitope specificity (e.g., avidity, immunoblot, virus  
444 neutralisation) and implement paired mother-fetus and/or mother-child testing, as was

445 recently reported for ZIKV neutralising antibodies [303]. Differences in background exposure  
446 to ZIKV and other flaviviruses will have an (age-dependent) effect on cross-reactivity and  
447 interpretation of protein-driven assays, such as IB or micro-array analysis. In these instances,  
448 CMI might be explored as alternative method to differentiate maternal from congenital ZIKV  
449 infection. Standardisation of (validated reference) methods is critical in order to compare  
450 different methods and might need (a) reference centre(s) to confirm acute infection. There is a  
451 plethora of studies describing potentially improved diagnostics for the TORCH complex  
452 infections, including ZIKV. Exploration of the broad range of published methods is important  
453 to improve diagnostic algorithms. In the meantime, it is essential to raise awareness among  
454 medical microbiologists and treating physicians about the limitations of the presently applied  
455 tests and algorithms, guiding protocol development for diagnostic testing of (novel) infections  
456 such as ZIKV and optimise diagnostic algorithms, for the different geographic and resource  
457 settings. Given the observed disconnect between the different pathogen specialist fields, we  
458 conclude that there is a clear case to be made for an integrated TORCHeZ diagnostic  
459 challenge.  
460

461 **FIGURE LEGENDS**462 **Figure 1:**

463 Seroprevalence, maternal infection risk, fetal transmission risk and congenital infection risk  
464 of the following selected infections: TOXO, PB19, Rubella, CMV and ZIKV.

465

466 Legend:

467 \* seroprevalence at childbearing age

468 \*\* IR/yr= maternal annual infection rate

469 \$ FTR= fetal transmission rate

470 CIR = congenital infection rate= number of congenital infections per 1000 live

471 births (TOXO) or per 1000 pregnancies (RV, CMV, PB19, ZIKV)

472

473 **Figure 2:** Routine maternal diagnostic methods: sensitivity and specificity, PPV, NVP

474 median point estimate +/- 95% CI

475

476 Legend:

477 Abbreviations: PPV: positive predictive value, NPV: negative predictive value, 95% CI: 95%

478 confidence interval

479 References figure 2:

480 TOXO (n=28): 48,99,113,134,143,150,153,160,161,162,163,165,170,171,175,177,179,

481 183,189,195,199,200,201,204,205,209,262,304

482 PB19 (n=14):29,36,53,79,84, 86,121,146,176,197,211,305,306,307

483 RV (n=9):59,108,137,149,151,186,204,304,308

484 CMV (n=24):23,53,92,101,112,128,145,148,155,156,159,166,169,173,178,190,196, 204,

485 ,206,250,265,281, 304,309

486 ZIKV (n=15):289,310,311,312,313,314,315,184,316,317,318,319,320,321,322

487

488 **Figure 3:** Routine fetal diagnostics methods: sensitivity and specificity, PPV, NVP median  
489 point estimate +/- 95% CI

490

491 Legend:

492 Abbreviations: PPV: positive predictive value, NPV: negative predictive value, 95% CI: 95%  
493 confidence interval

494 References figure 3:

495 TOXO (n=13): 33,43,126,154,164,209,212,226,227,228,230,323,324

496 PB19 (n=5): 79,213,220,222,223

497 RV (n=1): 323

498 CMV (n=10): 89,129,169,210,222,223,224,229,277,323

499

500 **Figure 4:** Routine neonatal screening methods: sensitivity and specificity, PPV, NVP median  
501 point estimate +/- 95% CI

502

503 Legend:

504 Abbreviations: PPV: positive predictive value, NPV: negative predictive value, 95% CI: 95%  
505 confidence interval

506 References figure 4:

507 TOXO (n=9): 174,201,212,228,234,246,248,249,325

508 PB19 (n=2): 36,85

509 RV (n=2): 233, 252

510 CMV (n=11): 102, 115,128,158,159,215,238,250,253,277,326

511

512

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## **FIGURES**