Serological survey on bovine viral diarrhea virus in man and evaluation of relation with Zika virus-associated microcephaly

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Abstract

Background: In 2015, an unprecedented epidemic of microcephaly occurred in Brazil. Preliminary observations suggested the involvement of cofactors in the etiopathology of Zika virus-associated microcephaly. Bovine viral diarrhea virus (BVDV) was identified in fetal samples with microcephaly, originating in the state of Paraíba, and two virus sequences, obtained from the amniotic fluid collected from mothers with babies affected by Zika and microcephaly, have been characterized as two different species of BVDV, types 1 and 2.
Aim: The involvement of BVDV as a co-factor in the etiopathogenesis of Zika virus-associated microcephaly was explored.
Methods: A serological screening using an ELISA test was undertaken to detect antibodies against BVDV among patients referred to the Central Laboratory of Natal, Rio Grande do Norte, encompassing microcephalic babies and their mothers, mothers and pregnants not associated with microcephaly and general patients as a control group.
Results: Two samples were positive out of 382 tested (0.52%). No specific relation with birth defects could be established.
Conclusions: The study might suggest serological evidence of BVDV in humans. Further studies and the application of improved diagnostic tests adapted to humans are necessary to clarify the epidemiological extent and impact of BVDV.
Keywords: Bovine viral diarrhea virus, Brazil, Cofactor, Microcephaly, Zika virus.

Introduction

Zika virus is the agent of a disease of African origin, first reported in the late 1940s in Eastern Africa, in Uganda in 1947 (*Dick* et al., 1952), characterized for a long time as a mild arthropod-borne infection, causing small outbreaks in Western Africa and Asia (1960s, 1970s). Zika virus disease is transmitted primarily by *Aedes* mosquitoes, the same vector carrying dengue, yellow fever, chikungunya, and Rift Valley viruses. The transmission is vectorial and assured by *Aedes aegypti* and *Aedes albopictus*. However, also sexual transmission occurs, and the virus may persist in semen for about 6 months (Foy *et al*., 2011). Symptoms are resumed as a simile-influenza clinical course, with various symptoms such as fever, muscular and articular pain, and cutaneous rash. Neurologic complications are mainly Guillain-Barré syndrome (higher incidence in Brazil and French Polynesia), meningoencephalitis, transverse myelitis, and facial paralysis. However, the major complication is microcephaly which affects fetuses. The increased diffusion of the Zika virus seems to be linked to the expansion of *A. albopictus*, commonly known as the Asian tiger mosquito, originally restricted to some Asian regions and Madagascar until 1980, then spread throughout the world, from Australasia, Europe, and to the Americas. This allowed a geographic extension of arboviruses. Zika virus reached naïve human populations, causing an epidemic in Micronesia in 2007 (Duffy *et al*., 2009). The spread was accompanied by the occurrence of more severe clinical forms. Similarly, this was also observed for the Chikungunya virus, until 2005, considered a self-limiting and non-fatal disease, responsible in 2005–2006 in La Réunion for a serious outbreak affecting 35% of the population (265,000 ill persons) and causing 254 deaths due to meningoencephalitis (Vazeille *et al*., 2007).

From Micronesia, the current strain of Zika virus diffused to Nouvelle Calédonie, French Polynesia, and other islands in the Pacific Ocean in 2013 and 2014 (Cao-Lormeau *et al*., 2016). In 2014, the virus was reported in Chile (Easter Islands), then in Brazil in 2015, and subsequently registered in numerous other countries of the Americas (Musso, 2015). All the isolates were

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genetically more closely related to that of Polynesia than the African strains (Calvez *et al*., 2018). In 2015 and 2016, Brazil lived an unprecedented epidemic of microcephaly that has not yet been fully explained (Heymann *et al*., 2016). Also, cases of Guillain-Barré syndrome increased dramatically. However, after 2 years, a radical decreasing trend occurred throughout Brazil. Similar decreasing trends of Zika infection and associated health complications, including Guillain-Barré syndrome, have been observed elsewhere, as in French Polynesia during 2013–2014 (Cao Lormeau *et al*., 2016).

Previous to Zika occurrence, microcephaly was endemic in Brazil at a low rate among the population, as demonstrated by a study conducted by the Faculty of Medicine of Ribeirão Preto and the Federal University of Maranhão (Grota, 2018). Therefore, the Zika virus represented an apparent risk factor determining the increase in cases. Furthermore, a cross-sectional study on the epidemic of microcephaly due to Zika virus in Brazil (Medeiros Figueiredo *et al*., 2021) indicated a higher risk of microcephaly linked to the infection. It provided evidence that cases of the Zika virus occurred well before the initially suspected introduction of the virus in the country, linked with the Olympic games during the summer of 2016. In reality, epidemiological evidence suggested the entry of a new virus, indicating the presence of Zika virus in Brazil between the second quarter of 2013 and the beginning of 2014, considerably increasing the risk of microcephaly. This also corresponds to observations made in the studied area. The first case of microcephaly was retraced in June 2014 in Lucrécia, a tiny municipality of Rio Grande do Norte, well documented by typical skin rash in mother and compatible neuro-images (de Andrade and Giangaspero, 2017).

However, the epidemiology of microcephaly associated with the Zika virus in Brazil suggested the occurrence of possible cofactors, suspecting an association with farmed animals. Dr. Marinho, director of information and health analysis at Brazil's Health Ministry, raised the question of something more than the Zika virus was causing the high intensity and severity of cases, suspecting that the cattle-farming industry may indeed be involved, and looking into the question of whether Bovine viral diarrhea (BVD) has combined with Zika to cause birth-defects (Butler, 2016; Mountain, 2016). Livestock is not known to be susceptible to the Zika virus. Still, livestock is kept in peri-urban and rural areas, where their need for water also increases opportunities for mosquitoes to breed and thrive. Nevertheless, recent research undertaken in 2018 by the Biological Institute, linked to the São Paulo Department of Agriculture, identified the presence of Zika virus in healthy cattle and deer in the interior of the State of São Paulo, suggesting that cattle can contribute to the spread of Zika virus (Okuda, 2019), but this did not explain the increased pathogenicity of Zika virus. On the other side, BVD affects cows worldwide, capable of interfering with central nervous system development in fetuses, causing cerebellar hypoplasia, hydranencephaly, internal hydrocephalus, microencephaly, or porencephaly, and it is known to express synergy with other pathogens in animal diseases.

Research teams from the Federal University of Rio de Janeiro and the research Institute Professor Joaquim Amorim Neto, Campina Grande, identified BVD virus (BVDV) in fetal samples with microcephaly, originating in the state of Paraíba (Nogueira *et al*., 2016). Two virus sequences obtained from the amniotic fluid collected from mothers with babies affected by Zika and microcephaly have been characterized as BVDV, according to secondary structure analysis (Giangaspero and Apicella, 2018). The two sequences were clustered among sub-genotype BVDV-1b1, of cosmopolitan diffusion, and BVDV-2b, a typical genotype circulating in South America, showing unreported variants in the Internal Ribosome Entry Site. The only strain previously reported in humans belonged to genotype BVDV-1d, another cluster with cosmopolitan diffusion (Giangaspero *et al*., 1997).

Aiming to explore the involvement of BVDV as a co-factor in the etiopathogenesis of Zika virus-associated microcephaly, a serological screening was undertaken to detect antibodies against BVDV in the human population in northeastern Brazil, an area severely affected by the outbreak of microcephaly.

Material and Methods

Samples
Three hundred eighty-two serum samples were available from the Central Laboratory, Natal, Rio Grande do Norte, Brazil. The samples included sera collected from 2015 to 2017 (15 collected in 2015, 278 in 2016, and 89 in 2017) from patients (337 females and 45 males) of age ranging from 16 to 43, referred to the laboratory from the province of Natal and all other provinces of the State, also for serological investigations on arboviruses (Zika, Dengue, and Chikungunya viruses). Some women were mothers of microcephalic babies (*n* 51). In addition, babies (21 males and 17 females, 1–3 years of age) suffering from microcephaly were also tested. Mothers with microcephalic infants were generally favorable for Zika virus antibodies (87.5%), while only 24.39% of microcephalic babies were Zika virus antibody positive. Samples included pregnant women (1–3 months to 6–9 months of gestation). Most of them showed clinical symptoms associated with active Zika virus infection during sampling: fever, cutaneous rash, itch, headache, myalgia, intercostal pain, arthralgia, periarticular edema, asthenia, nausea, vomiting, and diarrhea. The selected sera have been organized into different groups: mothers with microcephalic infants and microcephalic infants (*n* 89, all collected in 2017), pregnant women with clinical symptoms without microcephalic fetuses (*n* 51), women not in pregnancy

and without microcephalic infants (*n* 130) and general samples (*n* 112) (control group) (Table 1).

Serological test

An ELISA commercial kit (Median, South Korea) to screen anti-BVDV antibodies in bovine animals was used in the present study. The test included bovine hyperimmune positive and negative control (NC) and was executed following the manufacturer's recommendations. Plates were read by an ELISA reader at 450 nm. Test sera and NC optical density (OD) values were used to compute the S/N ratio value (sample OD/ NC OD mean) for cut-off determination. Sera with S/N < 0.7 were considered positive.

Statistical analysis

According to their association with microcephaly, pregnancy, and general samples, human sera were compared for BVDV infection rate proportions by computing chi-square statistics based on the deviations of the observed ratios from equality. These chi-square tests were based on one degree of freedom, using Chi-Square Test Calculator, Social Science Statistics (Stangroom, 2022).

Ethical approval

This research was approved by the Brazilian National Council of Ethics in Research, Brasilia (opinion number 2.590.914, April 10, 2018).

Results

Human samples collected from Rio Grande do Norte, Brazil, for serological screening for antibodies to BVDV (ELISA test) showed an overall positiveness of 0.52% (*n* = 2) out of 382 tested (Table 2). One positive serum was from a woman who was not pregnant and without microcephalic infants, collected in 2015, and the other was from a woman in the group of general samples collected in 2016. Based on the cut-off value of S/N < 0.7, valid for cattle, five sera were positive. These sera were retested, and three did not score positive SN value. Thus they were considered negative. Forty-nine sera (12.83%) showed low reactivity against BVDV antigen, just under the cut-off value (S/N >0.7/<0.9). Retesting did not confirm additional eight sera as low reactive, thus considered negative.

Adjusting the cut-off for positiveness to S/N < 0.9 for the application on human sera, taking into account the low antibody titers that humans develop against BVDV, especially cytopathic (CP) strains, generally used as antigenic substrate in tests designed for cattle (Giangaspero *et al*., 1988, 1993a, 1993b), and also considering these "low reactive" sera as positive, and negative the sera with S/N ratio values ≥0.9, the overall positiveness could be 13.35%. Based on the cut-off value of S/N < 0.9, the group of mothers with microcephalic infants scored the lowest level of seropositive (5.88%). 10.53% of microcephalic infants were positive. A similar rate (10%) resulted in the group of women, not in pregnancy and not associated with microcephalic infants. In the group of pregnant women with clinical symptoms without microcephalic fetuses, the positiveness was 7.84%. Higher positive rates were observed in the group of general samples (control group) (24.11%). Concerning the collection year, 2016 accounted for the highest number of BVDV-positive sera (15.47%). Samples collected in 2015 and 2017 showed lower seropositiveness, 6.67 and 7.86 %, respectively. The statistical tests applied to compare the groups associated with microcephaly (mothers and infants) with other groups demonstrated that there was no difference between the groups (*p*-value > 0.1).

Discussion

BVDV in humans was preliminary explored by a serological survey in Rio Grande do Norte. The serological reaction observed in the present study might suggest the circulation of BVDV in humans. Furthermore, the results corroborated the previous isolation of BVDV in microcephalic babies (Nogueira *et al*., 2016). Considering that Zika and dengue viruses belong to the family Flaviviridae and BVDV was previously classified in the same family, in the present study, the occurrence of an eventual cross-reaction was excluded by the preliminary screening for Zika virus, dengue made on the tested sera.

Concerning the origin of the detected antibodies against BVDV in human sera, no evidence of transmission between humans was revealed, and

Table 1. Human samples collected from Rio Grande do Norte, Brazil, for serological screening for antibodies to BVDV (ELISA test).

Serum sample category	Number	Age range	Year of collection
Mothers with microcephalic infants	51	18–43 years	2017
Microcephalic infants (M: 23; F: 18)	41	1–3 years	2017
Women not in pregnancy and without microcephalic infants	131	18–36 years	2015 (6)–2016 (125)
Pregnant women with clinical symptoms without microcephalic foetuses	51	16–41 years	2015 (4)–2016 (47)
General samples (M: 24; F: 88)	112	-	2015 (5)–2016 (107)
Total	386		

Four sera were not tested due to insufficient quantity (two male and one female babies born in 2017 and one female sampled in 2016).

Table 2. Results of serological screening for antibodies to BVDV (ELISA test) in human samples from Rio Grande do Norte, Brazil.

Overall results						
BVDV (ELISA)	**Number**	**%**	**Sample OD**	**Positive control OD**	**NCOD**	**S/N value**
Positive HR	2	0.52	0.871–1.092	−0.007 to −0.115	1.5905–1.686	0.54762653–0.68657655
Positive LR	49	12.83	1.117–1.417	−0.004 to −0.15	1.443–1.686	0.77408177–0.89875439
Negative	331	86.65	1.299–2.083	−0.004 to −0.15	1.443–1.7575	0.9002079–1.2354685
Total	382					

Details by sample groups					
	Mothers with microcephalic infants	**Microcephalic infants (M: 21; F: 17)**	**Women not in pregnancy and without microcephalic infants**	**Pregnant women with clinical symptoms without microcephalic foetuses**	**General samples (M: 24; F: 88)**
	Number (%)	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**
Positive HR	0	0	1 (0.77)	0	1 (0.89)
Positive LR	3 (5.88)	4 (10.53)	12 (9.23)	4 (.)	26 (23.21)
Negative	48 (94.12)	34 (89.47)	117 (90)	47 (.)	85 (75.89)
Total	51	38	130	51	112

Distribution by year (2015–2017)					
Year	**Number**	**Positive (%)**	**Positive HR**	**Positive LR**	**Negative**
2015	15	1 (6.67)	1	0	14
2016	278	43 (15.47)	1	42	235
2017	89	7 (7.86)	0	7	82
Total	382	51	2	49	331

OD: optical density; NC: negative control; S/N ratio value: test serum OD/NC OD mean (cut-off for positiveness S/N < 0.7; adjusted cut-off for positiveness S/N < 0.9); HR: highly reactive (S/N < 0.7); LR: low reactive (S/N >0.7/< 0.9).

the immune reaction was probably related to the circulation of the infection in domestic animals. However, it cannot be excluded that another source might be the contamination of biological products with BVDV. Contamination of live virus vaccines for human use by adventitious BVDV was previously reported (Giangaspero *et al*., 2001). Nevertheless, the *Pestivirus* sequences from amniotic fluid collected from mothers with babies affected by Zika virus and microcephaly and characterized as BVDV-1 and BVDV-2 (Giangaspero and Apicella, 2018), were different from Asian BVDV strains detected in measles, mumps, and rubella vaccine samples used in Brazil (Prof Tanuri, Federal university, Rio de Janeiro, personal communication). In Central and South America, the serological positiveness for BVD in a human was previously reported only in one study undertaken in 1988, using indirect immunofluorescence and ELISA tests with non-cytopathic (NCP) A19 and CP C24V and NADL strains (Giangaspero *et al*., 1988). Potential cross-reactivity due to genetically related viruses such as classical swine fever virus, rubella, and yellow fever was considered and excluded.

The role of pestiviruses in human pathology remains unknown, and data on epidemiology in humans are still fragmentary. BVDV has been suspected as a co-factor in human immunodeficiency virus (HIV) infection. Seropositivity to BVDV was found in HIV-positive patients in a study undertaken in Zambia and Europe (Giangaspero *et al*., 1993a, 1993b). Furthermore, considering the tropism for nerve cells in animal pathology, pestiviruses have been suspected to be related to neurological disorders and birth defects in humans by different authors. In Italy, high anti-BVDV antibody titers were detected for 4 years in a farmer suffering from a rare form of post-viral mononeuritis of unknown etiology, the Personnage Turner syndrome (Di Bella, 1986), which arose at the same time as a fatal outbreak of BVDV in his cattle farm (Giangaspero and Cominardi, 2006). In the USA, antibodies against

BVDV were detected in mothers with microcephalic infants (Potts *et al*., 1987) and in 40% of sera from identical twins discordant for schizophrenia (Yolken *et al*., 1993). Infection during pregnancy and cerebral white matter damage in preterm neonates have also been explored (Dammann and Leviton, 1998, 2004; Leviton *et al*., 2005; Dammann *et al*., 2006; Rennie and Peebles, 2006). Most of the studies were based on serological screening, and some have been conducted to reveal antigens. However, these studies remained experimental, and no definite conclusions could be achieved. Similarly, despite the suspicion of co-factor concurrence in microcephaly, no relation could be established in the present study between BVDV and Zika virus-associated birth defects. Even taking into account results of sera close to positivity (*n* 49), while some of these "low reactive" sera were also observed among infants affected with microcephaly and their mothers, seroconversion in this group associated with microcephaly was low. Only in one case were the mother and the respective child positive for Zika virus antibodies and low reactive to BVDV. In addition, low reactive sera were also distributed independently in all other patient groups unrelated to microcephaly.

Concerning serology, the main problem with conducting surveys on the zoonotic potential of pestiviruses is related to the lack of specific tests to be applied to human samples. In the present study, due to diagnosis limitations, there is a remote chance that the two positive cases were false positives. However, the sera were retested, and only those showing high OD values were retained as positive. Nowadays, no commercial test for BVDV in humans is available. Therefore, diagnostic tools validated for cattle were considered an alternative to being possibly applied as routine studies as serological surveys on human samples instead of more complex and expensive homemade tests. Using kits designed for bovines represents a major problem regarding antigen substrate and different immune responses seen in humans and cattle. According to previous studies, the immune reactivity of human sera against BVDV strains showed considerable variation. Human sera showed much lower titers against BVDV compared to those obtained with bovines, and this difference was enhanced when using CP strains. Human sera scored higher antibody titers with NCP strains and low titers or negative with CP strains as NADL, commonly used as antigen substrate in commercial ELISA kits for cattle screening (Giangaspero *et al*., 1988, 1993a, 1993b). Of course, this aspect represented a limitation of the present study, as the optimal should have been the application of a validated test for humans, implying the use of NCP BVDV strains as antigenic substrate, reported more appropriate to reveal immune reactions against pestiviruses in man. The not negligible number of sera-scoring results just behind the S/N cut-off value valid for cattle (about 12.8%) recall this need for future studies.

Conclusion

Although the study could not contribute to elucidating causes and co-factors that determined the severe epidemic of microcephaly in Brazil, the serological results obtained on human sera screened for anti-BVDV antibodies represented a preliminary activity in the framework of investigations on *Pestivirus* diffusion in the human population. They might suggest a circulation of BVDV in men in northeastern Brazil. More sensitive and confirmation methods have to be applied in future studies. Antigenic evidence will be fundamental through isolation and sequencing. Progress has been made in clarifying the pathogenesis of Zika virus infection (Wen *et al*., 2017). However, many unanswered questions remain regarding risk factors and epidemiology (Dufort and White, 2018). The epidemic of microcephaly in Brazil has to be fully understood to allow efficient prevention and control measures against the reoccurrence of the phenomenon, also elsewhere. Other pathogens might be considered in further studies focusing on potential co-factors in the etiopathogenesis of Zika virus-associated microcephaly. Among pathogens of animal origin, the Schmallemberg virus, transmitted by mosquitoes, causing abortion and birth defects in cattle and small ruminants, was considered and ruled out since no evidence could be observed among domestic animals in Brazil (de Souza Nunes Martins *et al*., 2022). Furthermore, other host species with the potential to play an epidemiological role in Zika virus disease should be investigated. An extensive serological survey among horses from New Caledonia and French Polynesia demonstrated that horses could be infected with the Zika virus (Beck e*t al*., 2019).

Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contribution

M. Giangaspero and T. Okabayashi contributed equally to the present study.

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