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VI, 1. Epidemiology of toroviruses

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The toroviruses

The discovery of the toroviruses, which comprise a genus of the family Coronaviridae, began with the serendipitous isolation of the morphologically unique Berne virus (ETV) in feces of a horse (Weiss et al., 1983). This was followed by the discovery of the Breda virus (BoTV), a morphologically similar enveloped virus particle, which was detected in, but not isolated from the stools of diarrheic calves (Woode et al., 1982). Breda viruses were subsequently found to exist as two antigenically distinct serotypes, referred to as BoTV-1 and BoTV-2 (Woode et al., 1985). Morphologically similar pleomorphic particles characterized by a well described fringe around the outer edges were observed in human stool specimens (Beards et al., 1884; Duckmanton et al., 1997). These have been generally referred to as torovirus-like particles or coronavirus-like particles (Mortensen et al., 1985). More recently, a porcine torovirus (PoTV) has been found and partially characterized (Kroneman et al., 1998). There is serological evidence that toroviruses are present in several other animal species (Weiss et al., 1984).

Biological basis for torovirus-specific diagnostic assays

Investigations of the epidemiology of toroviruses necessitate having tests for the detection of the virus, its antigens and its nucleic acids in clinical specimens and having well defined antigens for tests to detect the immune response to the virus. ETV was first detected by the isolation of the virus in cell culture (Weiss et al., 1983). Growth of ETV in cell culture allowed for the characterization of the virus, in terms of its morphology, morphogenesis, protein and nucleic acid composition, and facilitated investigations into its replication strategy (Weiss and Horzinek 1986; Horzinek et al., 1987; Snijder et al., 1988). These studies demonstrated that the virus has a unique morphology with a helical nucleocapsid in a torus configuration surrounded by an envelope containing a spike protein, S, and a trans-membrane envelope protein, E. The nucleocapsid is composed of the N protein and a single stranded positive sense
RNA genome. The sequence of the genome was shown to encode 3 structural proteins, N, M and S and to possess a pseudogene subsequently shown to be homologous to the gene which encodes the HE protein of BoTV (Snijder et al., 1993). Studies on the viral replication in cell culture revealed that the virus proteins are translated from nested, 3’-coterminal mRNAs (Snijder et al., 1988). This information, along with the viral structure and nucleic acid sequence established that toroviruses are a genus of the family Coronaviridae. (Cavanaugh, 1997)

After the description of the characteristic morphology of ETV, torovirus-like particles were readily identified by electron microscopy (EM). Unfortunately, the pleomorphic nature of the viruses makes EM for this virus less definitive than, for example, in the case of rotavirus or even coronavirus which have very well defined structural features. The major criteria for the EM diagnosis of torovirus include a characteristic fringe of spike proteins on the periphery of the particle, a diameter of approximately 100 nm and a subset of particles having a comma shaped appearance (Weiss et al., 1983; Duckmanon et al., 1997). Since stool specimens can contain a wide assortment of particulate matter, it is difficult to unambiguously identify torovirus-like particles in such preparations. Accordingly, for definitive EM diagnosis the presence of virus should be confirmed by IEM with a reference antibody to torovirus (Beards et al., 1984; Duckmanon et al., 1997).

The successful propagation of ETV in cell cultures facilitated the development of a virus neutralization assay, which made it possible to gain an insight into the sero-epidemiology of this virus in various animal populations (Weiss et al., 1984). In contrast, no other torovirus has as yet been isolated in cell culture, which has been a significant impediment to the investigation of these agents. A bovine respiratory torovirus was reported to have been isolated from calves but has since been shown to be a coronavirus (Cornelissen et al., 1998). ETV was further shown to hemagglutinate rabbit and guinea pig and human group O erythrocytes (Zanoni et al., 1986). BoTV purified from calf stools was shown to agglutinate mouse and rat erythrocytes (Woode et al., 1982). The hemagglutination is believed to be mediated by the S protein of ETV and the HE protein of the BoTV virus. These findings have allowed for the development of the hemagglutination inhibition assay as a more versatile serological test to follow the sero-epidemiology of these viruses (Jamieson et al., 1998).

Specific antibodies to BoTV from infected gnotobiotic calves and purified BoTV were used in the development of ELISAs for the detection of viral antigens and antibodies, respectively (Brown et al., 1987, Woode et al., 1985, Koopmans et al., 1993). The preparation of reference reagents for BoTV was facilitated by purification of virus from stool specimens of gnotobiotic and then infected calves (Woode et al., 1985). Reference antisera to BoTV were also adapted to identify these viruses by immune electron microscopy (IEM) (Beards et al., 1984). These assays facilitated studies on virus shedding and serological responses and also showed that antigenic cross-reactivity exists between these viruses. Finally, when the partial sequence of the genomes of ETV and BoTV were obtained (Snijder et al., 1993; Cornelissen et al., 1998; Duckmanon et al., 1998b), it became possible to detect these viruses in stool specimens by hybridization or by RT-PCR (Koopmans et al., 1991a; Duckmanon et al., 1998b).
Epidemiology of ETV

Although ETV has been isolated only once from a horse, there is evidence that antigenically related viruses are widespread in the equine population (Weiss et al., 1984). The sero-prevalence of ETV-specific antibody as measured by virus neutralization test is very high in the horse population with 35% of randomly collected sera in Germany (Liebermann, 1990) and 81% in Switzerland being positive (Weiss et al., 1983, 1984). There is further evidence that the virus is actively circulating since 9% of 273 horses examined experienced sero-conversion when sequential sera separated by 3 to 45 days were tested for the presence of antibody (Weiss et al., 1984). Neutralizing antibody was also found in sera from cattle, goats, sheep, pigs, rabbits and mice but not in veterinarians. These early studies provide an important insight into the prevalence of ETV or antigenically closely related viruses among different animal species.

Epidemiology of BoTV

Testing for antibody to BoTV has been performed by ELISA using virus purified from stools of infected calves as antigen (Woode et al., 1985). Screening of sera from calves in the U.S.A. and Germany, showed that 88.5 and 75%, respectively, were found to be reactive for antibody to BoTV by this method (Woode et al., 1985; Liebler et al., 1992). Likewise, cattle in the UK and the Netherlands were found to be sero-positive for antibody to BoTV (Brown et al., 1987; Koopmans et al., 1989). Overall, it has been estimated that 90-95% of random serum samples from cattle are reactive for antibody to either ETV or BoTV (Koopmans and Horzinek, 1994). Electron microscopic surveys of stool specimens from diarrheic calves in France showed that 76% contained torovirus-like particles (Lamouliatte et al., 1987). These early findings showed that BoTV is widespread in cattle over geographically diverse locations.

Evidence for the role of BoTV in gastroenteritis

The definitive etiological role of BoTV in diarrhea was established by the inoculation of germ-free calves with Breda virus (Pohlenz et al., 1984). The calves became symptomatic and shed an abundant amount of virus. Studies on infected calf tissues by electron and immunofluorescence microscopy demonstrated that the sites of infection were predominantly in the crypt cell area of the gut. A study on sentinel calves showed that the animals readily became infected with BoTV from a contaminated environment and continued to shed the virus for a period of 4 months (Koopmans et al., 1990). During this time virus was eliminated from a subset of calves but on re-introduction into the herd they became re-infected.

Studies on two populations of cattle, breeding calves up to 2 months of age and adult cattle experiencing dysentery, showed that virus shedding, measured by ELISA, occurred in 6% of symptomatic calves, and torovirus seroconversion was manifest in
over 40% of adult cattle with dysentery (Koopmans et al., 1991c). In a survey of stools from diarrheic calves collected from Canadian farms, BoTV was found by both EM and RT-PCR in 27.9% of symptomatic animals compared to 11.6% of asymptomatic controls (Duckmanton et al., 1998a). These findings demonstrate that BoTV is associated with diarrhea in infected cattle and that such infections are common. To what extent toroviruses are associated with diarrhea in other animals is unknown. In the case of piglets, virus shedding can be detected within a month of age, but there is no firm evidence that this is associated with disease (Kroneman et al., 1998). Likewise, foals experimentally inoculated with ETV remained asymptomatic despite manifesting a rise in neutralizing antibody (Weiss et al., 1984).

Transmission of BoTV

It is generally believed that torovirus spreads in cattle by the oral-fecal route. This is supported by the high concentrations of virus (up to 10\textsuperscript{11} particles per mL) shed from experimentally infected calves (Koopmans and Horzinek, 1994). Hence in an outbreak situation, the environment is likely to contain substantial amounts of virus, which would place many animals at risk of infection. However, it has been reported that toroviruses may also spread by the airborne route (Vanopdenbosch et al., 1992). Specifically, BoTV has been found in the respiratory tract of calves. Likewise, calves were successfully infected with BoTV by the intranasal route (Pohlenz et al., 1984). Finally, young calves, which were shown to seroconvert also manifest respiratory symptoms (Koopmans et al., 1989). In view of the report that some of these viruses from the respiratory tract which grow in cell culture are in fact coronaviruses, the respiratory route of transmission of torovirus may be open to question (Cornelissen et al., 1998). This issue has to some extent been resolved by the recent molecular studies on BoTV as described below (Hoet et al., 2002).

Epidemiology of human torovirus

Preliminary evidence of the existence of a human torovirus came from the reports of finding torovirus-like particles by EM in stool specimens of symptomatic patients which were shown to be reactive on IEM with BoTV specific antiserum and human convalescent serum (Beards et al., 1984). These findings were augmented by the report that coronavirus-like particles were the sole identifiable entity found in a very high number of stool specimens of patients with gastroenteritis (Mortensen et al., 1985; Payne et al., 1986). Because of the pleomorphic appearance of these particles, when viewed by electron microscopy, there is justifiable concern whether these were indeed viruses as opposed to blebs of membrane from the mucosa or breakdown products of bacteria. Supportive evidence of their identity as torovirus was provided by the finding that 68% of stool specimens containing torovirus-like particles were reactive in an ELISA based on antibody to BoTV (Koopmans et al., 1993). Additionally, RNA
extracted from stool specimens in which torovirus-like particles were demonstrated by EM, was found to hybridize with cDNA probes derived from the 3' end of the ETV genome (Koopmans et al., 1991b). Hence there is evidence at the morphological, antigenic and nucleic acid level that supports the existence of the human torovirus.

The prevalence of human torovirus remains controversial. Initial sero-surveys with ETV neutralization tests and BoTV ELISA failed to detect an appreciable presence of antibody to these viruses in human sera (Weiss et al., 1984; Brown et al., 1986). This observation is at odds with reports from laboratories in which viral gastroenteritis diagnosis is performed by EM that a very high number of specimens containing torovirus-like particles can be detected (Mortensen et al., 1985). The presence of torovirus-like particles was shown to be associated with gastroenteritis in a case-control study in which 35% of symptomatic patients compared to 14.5% of matched controls were found to be shedding torovirus-like particles (Jamieson et al., 1998). In this setting, excretion of torovirus-like particles was found more commonly in immunocompromised patients and those with nosocomially acquired gastroenteritis. Likewise, in a case control study on childhood diarrhea in Brazil, torovirus was detected by ELISA based on BoTV antiserum in 27% of children with acute diarrhea, in 27% of children with persistent diarrhea but not in controls (Koopmans et al., 1997). While the former study was directed to hospitalized children, the latter addressed the role of virus in the community. A subsequent community and hospital based study showed that torovirus-like particles were detected in 6% of symptomatic patients (Waters et al., 2000). These studies provide supportive evidence for the existence of human toroviruses and an insight into its prevalence in different populations. However, there remains a need to establish definitive nucleic acid based assays to augment the sensitivity and specificity of diagnosing these viruses in clinical specimens.

**Molecular epidemiology of toroviruses**

The sequences of approximately half of the genome of the ETV and the 3’ end of the BoTV, which encode the structural proteins have been reported (Snijder et al., 1993; Cornelissen et al., 1997; Duckmanton et al., 1998b). It is therefore possible to apply RT-PCR to detect these viruses in specimens from infected animals with enhanced sensitivity and specificity. This approach has been exploited in the testing of cattle for the prevalence of BoTV. In a study of 112 calves with diarrhea, 43% were positive for BoTV by RT-PCR compared to 30% when tested by EM (Duckmanton et al., 1998). In a more recent study of feedlot cattle, BoTV was found to be shed in stools in 37% of 57 steers 6-7 month old when tested by ELISA and in 95% when tested by RT-PCR (Hoet et al., 2002). Furthermore, it was demonstrated that nasal shedding occurred in 27% of the cattle when tested by ELISA and in 100% by RT-PCR. This study serves as an excellent model for the application of RT-PCR to the detection of toroviruses in symptomatic animals and has promise for similar investigations in humans. It also provides us with new insights on the sites of shedding of these viruses and their mode spread. Based on the published genome sequences it is now possible to express viral structural
proteins of BoTV to produce well pedigreed supplies of antigens and antibodies for
the design screening tests for these viruses (Cornelissen et al., 1997; Duckmanton et al., 1998b). The further development of RT-PCR assays is critical to define the epide-
miology of these viruses, namely to establish their prevalence in different populations,
define their mode of spread, explore the possibility of animals being a reservoir for
human infection and definitively resolve their role as agents of human gastroenteritis.

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