Two Electropherotypes of Bluetongue Virus Serotype 2 from Naturally Infected Calves

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SUMMARY

The first isolation of bluetongue virus (BTV) serotype 2 in the U.S.A. was in 1982 from a sentinel herd of cattle at Ona, Florida. Electrophoretic analysis of genome RNA revealed that all 16 serotype 2 isolates obtained from this focus of infection had one of two electropherotypes (designated Ona A and Ona B). All genome segments of Ona A and Ona B had detectable differences in electrophoretic mobility, with major differences noted for segments 1, 4, 5, 7, 8, 9 and 10. Electrophoretic comparison revealed that Ona A was indistinguishable from the African serotype 2 prototype isolated 23 years earlier. In 1983, Ona B, in the apparent absence of Ona A, was isolated from two additional cattle herds in Florida. These results suggest that Ona B may be a variant of Ona A that evolved as a result of mutation or reassortment with another BTV strain, and may be better adapted to the selective pressures found in southern Florida. Comparison of the electropherotypes of Ona B with two Florida isolates of serotype 13 and 17 and the prototypes of BTV 10, 11, 13 or 17 produced no evidence for reassortment between Ona A and any of these strains as the possible origin of Ona B.

INTRODUCTION

Bluetongue virus (BTV), a member of the Reoviridae family, Orbivirus genus, is arthropod-borne, replicating in both Culicoides vectors and ruminant hosts. BTV has been isolated in the United States (Barber, 1979), the Indian subcontinent, throughout Africa (Howell, 1963), the Middle East (Gambles, 1949; Komarov & Haig, 1952; Sellers et al., 1979) and Australia (St. George et al., 1978). In addition, antibodies to BTV have been reported from the sera of domestic ruminants from the Caribbean (Gibbs et al., 1983a) and South America (Groocock & Campbell, 1982) although isolations have not been reported from these regions. At present, five serotypes of BTV, 2, 10, 11, 13 and 17, are known to exist in the United States (Barber, 1979; Gibbs et al., 1983b) The most recent serotype confirmed in the U.S.A. was serotype 2 which was isolated from samples collected in 1982 from cattle located near Ona, Florida. Sixteen viral strains were isolated from individual blood samples of cattle and Culicoides insignis Lutz located near Ona, Florida (Gibbs et al., 1983b; Greiner et al., 1985).

The genetic diversity of BTV, which has a dsRNA genome with 10 segments, has been analysed by PAGE (Verwoerd et al., 1979; Gorman et al., 1981; Knudson et al., 1982; Squire et al., 1983) and two-dimensional gel electrophoresis of RNase T1-digested RNA (Sugiyama et al., 1981, 1982; Collisson & Roy, 1983). These techniques have the advantage of providing information on all 10 genome segments, each coding for unique proteins (Gorman, 1979; Verwoerd et al., 1979) whereas serotyping assays identify primarily external proteins (Huismans & Erasmus, 1981; Kahlon et al., 1983). Genetic shifts apparently resulting from segment reassortment and genetic drift (point mutations) have been identified by analysis of oligonucleotides produced by RNase T1 digestion of individual segments (Sugiyama et al., 1981, 1982; Collisson & Roy, 1983). Single-dimension PAGE, although not providing conclusive evidence on sequence homologies, has
been a convenient method for comparing the genomes of the Reoviridae (Hrdy et al., 1979; Flewett & Woode, 1978; Rodger & Holmes, 1979; Gorman et al., 1981; Knudson et al., 1982; Squire et al., 1983). Squire et al. (1983) found that variations in genome electropherotypes of field isolates of BTV 10 and 11 depended on geographical and temporal distribution of the isolates. Although variations occur in electropherotypes of rotaviruses, Schnagl et al. (1981) found that predominant electropherotypes could be identified in rotavirus epidemics.

In the present paper, dsRNA of field isolates from cattle infected with BTV at three locations in Florida were examined by PAGE. Two distinct electropherotypes were identified. Temporal distribution of the two electropherotypes indicated a possible shift occurring in nature from one electropherotype to a second during the initial episode of BTV infection in Florida in 1982 and by the re-occurrence of the second in 1983. The origins of the two electropherotypes of U.S.A. serotype 2 were investigated by comparing the RNA profiles of the Florida strains with the African prototype of serotype 2 and with the U.S.A. prototype strains of BTV 10, 11, 13 and 17 and strains of serotypes 13 and 17 from Florida.

**METHODS**

**Viruses.** BTV field strains were isolated from heparinized blood samples collected from individual cattle. The isolations were made by intravascular inoculation of 11-day-old embryonated chicken eggs (ECE) (Foster & Luedke, 1968) followed by passage of infected embryos onto BHK-21 cells. The U.S.A. BTV prototype strains used were: serotype 10, strain 8, collected in California; serotype 11, Station strain, Texas; serotype 13, strain 67-41B, Idaho; serotype 17, strain 62-45S, Wyoming. Two strains of BTV isolated in 1967 in Florida, BTV serotype 13 strain BT(OX)-315(FL 13) and BTV serotype 17 strain 67-41B(FL 17), were also studied. The prototype for BTV serotype 2 was isolated from sheep in 1959 in the Republic of South Africa. All viruses were adapted to replicate in BHK-21 cells. Viruses for electropherotype determinations were plaque-purified three times but are representative of the Florida isolations.

**Serotype assays.** Viral isolates from Rainbow Lakes Estate and Belle Glade were assayed for serotype by the disk plaque inhibition test (Stott et al., 1978). Antigenic similarities between the U.S.A. serotype 2 electropherotypes were determined by the plaque neutralization test (Jochim & Jones, 1976).

**Preparation of viral RNA and proteins.** Virus-infected BHK-21 cells were suspended in 0.1 M-sodium acetate, 0.001 M-EDTA pH 5, lysed with SDS and extracted with a phenol mixture (Sugiyama et al., 1981). Proteins labelled with [3H]leucine (ICN Pharmaceuticals) were analysed from infected cell extracts (Kahlon et al., 1983).

**PAGE.** Labelled protein lysates and RNA were put on 1.5 mm, 10% acrylamide gels with 5% stacking gels (Laemmli, 1970). Gels were stained according to instructions with the Gelcode kit (Upjohn, Kalamazoo, Mich., U.S.A.) with minor modifications. Gels were washed with ethanol and acetic acid, stained with silver for 1.5 h, and rinsed thoroughly with distilled water for 2 to 3 min. The gels were then covered with developer for 5 min, rinsed, and fixed for 10 min in 5% acetic acid. After a final rinse, they were stored in water.

**RESULTS**

**PAGE analysis of the U.S.A. and African prototype strains of serotype 2**

The genomes of 17 Ona, Florida isolates including 16 from cattle blood and one from a pool of C. insignis were analysed by PAGE (Fig. 1). All the isolates were found to have one of two electropherotypes, designated Ona A and Ona B as shown in lanes (c) and (e), respectively. When co-electrophoresed (lane d), major differences were seen in the mobility of segments 1, 4, 5, 7, 8, 9 and 10. Small differences were also found in the migration of segments 2, 3 and 6. The diffuse bands between segments 1 and 2, and 9 and 10 are not part of the RNA electropherotypes since they were present in cell controls, not labelled in vitro with [32P] or [3H]uridine and were found in varying positions relative to the RNA segments.

The genome of the serotype 2 prototype, which had been isolated in 1959 in the Republic of South Africa (Fig. 1a), was also co-electrophoresed with the genome of Ona A (Fig. 1b). The RNA from the two viruses appeared to be identical under conditions that showed differences in all 10 segments of Ona A and Ona B.

**Protein analysis of the serotype 2 Ona A, Ona B, and the African prototype**

Protein analysis of Ona A, Ona B and the African prototype of serotype 2 along with the U.S.A. prototype of BTV 10 is shown in Fig. 2. Viral proteins are numbered according to the
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Fig. 1. Co-electrophoresis of the genome of BTV serotype 2, Ona A with the African BTV serotype 2 prototype and Ona B. (a) Electropherotype of the African BTV 2; (b) co-electrophoresis of the African BTV 2 and Ona A; (c) Ona A; (d) co-electrophoresis of Ona A and Ona B; (e) Ona B.

Fig. 2. Proteins from infected BHK-21 cell extracts labelled with [3H]leucine. (a) Cell control; (b) BTV 10-infected cells; (c) Ona B-infected cells; (d) Ona A-infected cells; (e) African BTV 2-infected cells.

designation of BTV 1 proteins by Sangar & Mertens (1983) as VP1 to VP7 and non-structural proteins are labelled as NS1, NS1a and NS2. Differences could be seen between the proteins of Ona A and Ona B. Non-structural protein 2 of Ona A migrated at a slower rate than the corresponding protein of Ona B, and VP7 of Ona A migrated faster than that of Ona B. There were no discernible differences in Ona A and the African serotype 2. The proteins of BTV serotype 10 differed in VP2 (the major external protein), VP5 and VP7 from Ona A and in VP2 and NS2 from Ona B. VP6 is apparently the faint band between NS2 and VP7. There were two protein bands between VP6 and VP7 labelled 6a which were seen in Ona A and the African prototype but not seen in Ona B and BTV 10. PAGE analysis of Vero ATCC cell lysates infected with the three serotype 2 strains was identical to the BHK-21 cell lysates (data not shown). The proteins of the African prototype virus were also indistinguishable from Ona A.
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Fig. 3. Co-electrophoresis of the RNA of Ona B with the U.S.A. prototypes of BTV serotypes 10, 11, 13 and 17. (a) Electropherotype of prototype of BTV serotype 10; (b) co-electrophoresis of Ona B and BTV 10; (c) Ona B; (d) co-electrophoresis of Ona B and BTV 11; (e) prototype of BTV 11; (f) prototype of BTV 13; (g) co-electrophoresis of Ona B and BTV 13; (h) Ona B; (i) co-electrophoresis of Ona B and prototype of BTV 17; (j) BTV 17.

Antigenic similarities

Plaque neutralization tests with Ona A and Ona B were done with antibody prepared against homologous virus and the second Ona strain. Sheep sera prepared against Ona A neutralized 80% of the p.f.u. of both Ona A and Ona B at a dilution endpoint of 320, and serum prepared against Ona B neutralized both viruses at a dilution endpoint of 80.

Comparisons of the U.S.A. BTV serotype 2 genomes and previously known U.S.A. BTV serotypes

Ona A appeared to have originated from a source similar to that of the African prototype virus; however, the origin of Ona B was still in doubt. Since a rapid shift of so much of the genome could be best explained by a reassortment of segments resulting from co-infection of the Ona A strain and another BTV serotype, the dsRNA of Ona B was co-electrophoresed with the genomes of the prototypes of the four indigenous U.S.A. serotypes 10, 11, 13 and 17 (Fig. 3). None of the U.S.A. prototype viruses of BTV appeared to be closely related to Ona B. All 10 segments of Ona B and serotype 13 migrated at different rates (lane g). Only segments 3 and 8 of serotype 10 (lane b), segments 3 and 9 of serotype 11 (lane d) and segment 9 of serotype 17 (lane i) had electrophoretic mobilities similar to Ona B.

The prototype strains of BTV serotypes 10, 11, 13 and 17 were isolated before 1968 from specimens in California, Texas, Idaho and Wyoming, respectively. These viruses might be expected to be very different from a virus isolated in 1982 in Florida. A virus indigenous to the
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Florida region would be more likely to be responsible for the occurrence of Ona B through reassortment. The genomes of two Florida isolates of BTV (collected in 1967), serotype 13 (FL 13) and serotype 17 (FL 17), were co-electrophoresed with the prototypes of serotypes 13 and 17, respectively (Fig. 4b, h). On comparing FL 13 with the prototype of BTV 13, only one minor difference was found in segments 6. Although the prototype of 13 had been isolated in a different part of the U.S.A., the two electropherotypes were, in fact, very similar by PAGE. The FL 17, however, was found to have major differences from the prototype virus in the migration of segments 1, 2, 4, 5, 7, 8 and 10 and a minor difference in 6. When compared with Ona B, only electrophoretic mobilities of segments 6 of FL 13 and Ona B were similar. Segments 1, 4 and 9 of FL 17 resembled those of Ona B. With the limited similarities shown by PAGE, however, neither of the Florida isolates which we had at hand could account for the major differences between Ona A and Ona B.

Distribution of Ona A and Ona B

BTV serotype 2 was isolated from blood of cattle collected in 1983 at two sites in Florida other than the Ona site. The distribution of the Ona A and Ona B strains of BTV from the three episodes are summarized in Table 1. Ona A strains were isolated from seven blood samples collected at Ona in September and five in October, 1982. Ona B strains were collected at Ona
Table 1. Distribution of BTV serotype 2 and the electropherotypes isolated in Florida

<table>
<thead>
<tr>
<th>Location</th>
<th>Date</th>
<th>Ona A</th>
<th>Ona B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ona</td>
<td>1982 September</td>
<td>7*</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Rainbow Lakes Estate</td>
<td>1983 August</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>(near Dunnellon)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belle Glade</td>
<td>1983 September</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

* Number of isolates from individual cattle.

from three blood samples in October and from one in November. It was also of interest that two out of five animals from which virus was isolated in September and October bleedings apparently showed a shift from a population of predominantly Ona A in September to one of Ona B in October. Neither precipitating nor neutralizing antibody was detected in sera from these two animals until November (W. P. Taylor, personal communication). Ona A was also isolated from a pool of parous C. insignis collected in the Ona region in October (Greiner et al., 1985).

Fourteen strains of serotype 2 were isolated from individual cattle from Rainbow Lakes Estate which is approximately 125 miles northeast of Ona. Seven strains of serotype 2 were isolated from individual cattle at Belle Glade about 100 miles southeast of Ona. The electropherotype of all 21 viruses from these two sites was Ona B.

DISCUSSION

The electrophoretic analysis of the genomes of the first isolates of U.S.A. BTV serotype 2 from infected cattle is the first documentation of a natural shift of BTV from one electropherotype to a second in a single episode of infection. Each strain had a unique RNA and protein profile although both were serotype 2. Differences in proteins NS2 and VP7 were obvious. According to Sangar & Mertens (1983) and Grubman et al. (1983), who used BTV 1 and 17, respectively, the protein at the NS2 position is coded by genome segment 8 and VP7 by segment 7. Major differences were found between Ona A and Ona B strains in both these genome segments. Both viruses appeared to be stable forms of serotype 2. The electropherotype of the Ona A strain of BTV was indistinguishable from the African serotype 2 although they were separated by 23 years and several thousand miles. The Ona B strain was isolated from three locations over a 2-year period.

Assuming Ona B is a variant of Ona A, Ona B may have evolved as a combination of mutational events including point mutations and reassortment through a natural co-infection with Ona A and another BTV. The small differences in segments 2, 3 and 6 may suggest a mechanism of genetic drift; however, such a magnitude of difference in the electropherotypes seems to also favour a reassortment of genome segments. Ona B did not appear to be derived from any of the previous U.S.A. isolates of BTV examined. However, they are geographically and/or temporally removed from Ona B and may be poor representatives of more recent populations of BTV in the U.S.A. Alternatively, the second parental virus could be indigenous in the nearby Caribbean region.

Comparisons by PAGE of electropherotypes can be misleading especially when the total electropherotype is very different. Caution in interpreting electropherotype comparisons was emphasized by Clarke & McCrae (1982) who found by fingerprint analyses that segments with similar mobilities from different electropherotypes could have very different RNA sequences. While differing mobilities seemed to dictate differences in RNA sequences, such segments could still have highly conserved regions. A better understanding of the mechanisms responsible for the appearance and maintenance of Ona B in the BTV population in Florida depends on more extensive analysis of the genomes and proteins of the U.S.A. BTV serotype 2 viruses. PAGE analysis, while a valuable tool in characterizing populations of isolates, is limited as to the extent of biochemical diversity that it can identify.
The electrophoretic data on these three episodes of BTV infection in cattle have provided valuable information as to the epidemiology of BT. The similarity of 12 of the strains of serotype 2 to the African prototype strongly suggests that serotype 2 was recently introduced into the U.S.A., rather than having evolved from the four serotypes that were already present. The virus could have been introduced indirectly, perhaps by way of the Caribbean or South America, or directly, by way of apparently uninfected zoo animals imported from Africa. Another possibility is introduction by way of infected Culicoides spp. from the Caribbean; such movement of infected vectors was the probable mechanism for introduction of BTV into Cyprus (Sellers et al., 1979). Although the genome of BTV 2 can remain highly conserved, under certain circumstances relatively rapid genetic variation can apparently occur. The sequence of isolation events described for the U.S.A. BTV serotype 2 strains suggests the presence of environmental pressures which have selected for the electrophoretically distinct Ona B virus from a population which was originally stable for Ona A. Such selection pressures could result from changes in biological, biochemical or physical environments.

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REFERENCES


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