In recent years increased attention has been focused on infections caused by isolates of verocytotoxin-producing Escherichia coli (VTEC) serotypes other than O157. These non-O157 VTEC isolates are commonly present in food and food production animals. Easy detection, isolation, and characterization of non-O157 VTEC isolates are essential for improving our knowledge of these organisms. In the present study, we detected VTEC isolates in bovine fecal samples by a duplex 5' nuclease PCR assay (real-time PCR) that targets vtx1 and vtx2. VTEC isolates were obtained by colony replication by use of hydrophobic-grid membrane filters and DNA probe hybridization. Furthermore, we have developed 5' nuclease PCR assays for the detection of virulence factors typically present in VTEC isolates, including subtypes of three genes of the locus of enterocyte effacement (LEE) pathogenicity island. The 22 assays included assays for the detection of verocytotoxin genes (vtx1, vtx2), pO157-associated genes (etxA, katP, espP, and etpD), a recently identified adhesin (saa), intimin (eae, all variants), seven subtypes of eae, four subtypes of tir, and three subtypes of espD. A number of reference strains (VTEC and enteropathogenic E. coli strains) and VTEC strains isolated from calves were tested to validate the PCR assays. The expected virulence profiles were detected for all reference strains. In addition, new information on the subtypes of LEE genes was obtained. For reference strains as well as bovine isolates, a consistent relationship between subtypes of the LEE genes was found, so that a total of seven different combinations of these were recognized (corresponding to the seven subtypes of eae). Isolates with 15 different serogroup-virulence profiles were isolated from 16 calves. Among these, 53% harbored LEE and 73% harbored factors carried by the large virulence plasmid. One LEE-negative isolate had the gene for the adhesin Saa. The most common virulence profile among the bovine isolates was vtx1, eae-zeta, tir-alpha, etx,4, and espP. This panel of assays offers an easy method for the extensive characterization of VTEC isolates.