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**BRIEF COMMUNICATIONS**

Toxigenic *Corynebacterium diphtheriae* associated with an equine wound infection

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*Corynebacterium diphtheriae* is the primary etiologic agent of diphtheria, an acute communicable disease manifested by both local infection of the upper respiratory system and systemic effects induced by a toxin.\(^{1,9}\) *Corynebacterium diphtheriae* is a gram-positive, nonmotile, noncapsulated, aerobic, club-shaped bacillus that is classified into 4 bio-types: *gravis, mitis, intermedius,* and *belfanti.*\(^{7}\) Toxigenic strains of *C. diphtheria* are lysogenic for 1 of a family of corynebacteriophages that carry the structural gene for diphtheria toxin (tox).\(^{16}\) The toxin is the primary virulence factor of *C. diphtheriae.* Only *C. diphtheriae, C. ulcerans,* and *C. pseudotuberculosis* are known to harbor the phage-borne gene for production of the toxin.\(^{16}\)

Although *C. diphtheriae* is traditionally considered a non-zoonotic pathogen,\(^{7,11}\) isolation of *C. diphtheriae* from animals has been reported.\(^{5,10}\) *Corynebacterium diphtheriae* is an infrequent cause of bovine mastitis and has been associated with dermatitis with pyrexia in cattle. It is thought to be transferred to cows from infected dairy workers.\(^{5}\) *Corynebacterium diphtheriae* has also been documented in other domestic animals including equids and canids.\(^{6}\) French veterinarian Gaston Ramon was the first to develop and use diphtheria toxoid to treat the disease in the early 1900s,\(^{5}\) and documentation of the isolation of *C. diphtheriae* from equine wounds in Great Britain was reported in 1926,\(^{10}\) In addition, in the mid-1920s it was discovered that many horses carried “naturally occurring” diphtheria antitoxin.\(^{2}\) Within approximately 10 years after the end of World War II, following human diphtheria epidemics in France and Great Britain, the proportion of horses with circulating anti-diphtheria antibody decreased from approximately 71% to 5.6% and 16.7%\(^{14}\) In the 1960s, a Romanian abattoir study of equids presented for slaughter at the end of a diphtheria epidemic revealed that 6.92% of the 246 animals were colonized with *C. diphtheriae.\(^{14}\)

In the prevaccine era, diphtheria was one of the most common causes of death among children. The number of reported cases of diphtheria dramatically declined worldwide following the introduction of the diphtheria vaccine in the mid-1920s. Nevertheless, diphtheria remained endemic in at least 1 area of the United States until the 1970s\(^{12}\) and worldwide. In 1971–1981, there was a resurgence of diphtheria in the USA, and 7 outbreaks with 15 cases or more occurred. In addition, the long-term persistence of a particular clonal group of *C. diphtheriae* over the past 20 years in South Dakota has recently been documented.\(^{12}\) However, although the number of cases in the USA has significantly declined, studies of diphtheria immunity levels among US adults have shown that many adults (20–90%) do not possess adequate immunity against the disease.\(^{1}\) The reemergence of a susceptible population, in combination with circulation of toxigenic strains, could result in a resurgence of the disease. In fact, the waning immunity of a previously immunized population was considered to be a major factor for the recent diphtheria epidemic in Russia and the New Independent States (NIS) of the former Soviet Union in the early 1990s.\(^{1,4,13,15}\)

Here, we describe the isolation of toxigenic *C. diphtheriae* from the infected wound of a horse. The isolation of toxigenic *C. diphtheriae* from a horse reiterates the possibility of horses and other animals serving as reservoirs for *C. diphtheriae* in the USA and potentially playing a role in the circulation of toxigenic strains.

A 16-year-old Thoroughbred mare from a large equine breeding farm in semirural Virginia had foaled 8 months prior to first diagnostic evaluation. Approximately 10 other horses were pastured with the mare. The pastured animals were brought to a barn daily to be fed in stalls that were also routinely occupied overnight by another 10 mares with foals. The mares with foals were turned out each day into another pasture but shared common feeding troughs, water troughs, and stall space with the group containing the affected mare. The total composition of the group varied from time to time as mares foaled and were moved by their owners to other pasture areas. The total population of approximately 20–30 horses was routinely tended by the same farm workers and private veterinary staff.

The affected mare had difficulty foaling in both 1996 and 1997. On April 2, 1997, difficult foaling resulted in considerable postpartum hemorrhage, colic, and inability of the mare to stand for several hours. One week after foaling, the

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mare had an elevated temperature of 40 C and was treated with systemic and intrauterine aminoglycosides. During the course of the following illness, the mare lost 91 kg within 4 weeks of foaling and, despite a good appetite, became extremely cachectic. Within the month, a mucopurulent vaginal discharge developed and was treated with uterine lavage and systemic antibiotics, but the mare continued to deteriorate. Approximately 2 months after foaling, the mare developed what was first thought to be a puncture wound of the chest, possibly the result of a foreign body injury. Upon exploration of the chest wound, however, the attending veterinarian determined that the wound extended perhaps up to 30.5 cm into the chest muscle without evidence of a foreign body.

On May 29, 1997, the mare was admitted to the Marion DuPont Scott Equine Medical Center, where her treatment continued. On admission, the mare had joint inflammation and edema of the hind limb metatarsals, and the tarsi had bilateral effusion. Auscultation of the heart indicated a soft, blowing holosystolic murmur (I/IV) over the pulmonic outflow. Lungs were normal at rest. Debridement of the chest wound resulted in release of a copious amount of white exudate, and exploration of the wound demonstrated no foreign body. Vaginoscopic exam revealed pooling of a white exudate caudal to the cervix and within the right vaginal fold. Streptococcus zooepidemicus and Enterobacter agglomerans were isolated from this material. Hysteroscopy showed an enlarged, doughy right uterine horn with a large pedunculated cyst at the junction of the corpus and the uterine horn. Uterine biopsy revealed histologic evidence of acute moderate hemorrhagic endometritis and endometrial gland hyperplasia, with numerous cysts containing small numbers of bacterial rods. A complete blood count was normal, as was the fibrinogen level (300 mg/dl; normal range = 100–600 mg/dl), but plasma protein concentration was elevated (8.2 g/dl; normal range = 5.5–7.9 g/dl). Plasma protein electrophoresis showed hypergammaglobulinemia consistent with a chronic inflammatory process. Abdominocentesis revealed a high normal white cell count and protein of 1,000 mg/dl. There was no antibody response to Borrelia burgdorferi, causative agent of Lyme disease, and ACTH concentration was normal at 12.9 pmol/liter (normal range = 4.4–22 pmol/liter).

Treatment was instituted that included a daily uterine infusion with 1 liter of 30% dimethyl sulfoxide and 1 g gentamicin. Hind limbs were cleansed daily and treated daily with hydrotherapy and wrapping of the metatarsals. Procaine penicillin G (9 x 10^6 IU) was given intramuscularly twice daily for 5 days. Upon arthrocentesis of the tarsal joints, synovial fluid was serosanguinous, with protein of 4.6 g/dl (normal range = 0.6–2.0 g/dl) and a white blood cell count of 3,080 cells/µl (normal <500 cells/µl). After 5 days of treatment, uterine tone had improved dramatically, but formation was similar to urine pooling defect, and it was thought that the uterus was continually being reinoculated by pus draining from the caudal vagina. The mare was released from the facility with a guarded prognosis for a future pregnancy.

After discharge from the equine center, the mare was treated with Chinese herbal medications, which are generally used as feed additives, by personnel at the Middleburg Equine Clinic. These herbal preparations are now being examined for both specific and nonspecific immunomodulatory effects. Fractionation of some of these herbal preparations has shown antiinflammatory, antineoplastic, mitogenic, and a wide range of other defineable immunostimulatory activities. Although there was no healing of the original suppurating tract, the mare improved substantially in overall health and appeared clinically well except for the draining chest wound. The suppurative chest wound drained continuously for 8 months, and the horse was turned out to pasture.

On January 26, 1998, the thoracic exudate was cultured at the Virginia Department of Agriculture and Consumer Services, Warrenton Regional Animal Health Laboratory, and 3 organisms were isolated from the draining wound. There was a light growth of Streptococcus equisimilis, C. diphtheriae biotype gravis, and an Actinomyces sp. The C. diphtheriae colonie isolates were urease negative, catalase positive, and nitrate positive and fermented glucose, ribose, maltose, and glycogen. On staining with new methylene blue, swellings in the bacterium containing metachromatic granules were visible.

The C. diphtheriae biotype gravis isolate (strain A11) was first sent to the Virginia Department of Health Reference Laboratory for verification of identification. The isolate was then sent to the Diphtheria Reference Laboratory of the Centers for Disease Control for determination of toxigenic status. The A11 isolate was positive for both the A and B diphtheria toxin gene, tox, by polymerase chain reaction (PCR) (Fig. 2). The Elek assay that detects the presence of the diphtheria toxin also was positive (Fig. 3). Ribotyping was carried out as described previously. The A11 strain was unlike any toxigenic or nontoxigenic strain found recently in the USA and was unlike strains frequently isolated from the 1990–1996 diphtheria epidemic in the Russian Federation and the NIS (Fig. 4).

On April 15, 1998, an investigational follow-up visit to the breeding farm was arranged with the Virginia Department of Health so that cultures could be taken from the farm’s employees. Nine individuals agreed to have throat swabs evaluated by the Public Health Department for the presence of C. diphtheriae. A second culture of the mare performed during the April visit showed the C. diphtheriae was still present in the draining wound. On May 13, 1998, the mare foaled again, producing a healthy filly. On that day, cultures were taken again from the mare and from other horses and foals that were pastured and stabled with the mare. Environmental cultures were also taken, including stable materials, feeding and watering troughs, and common pasture areas. To determine the presence or absence of C. diphtheriae and related strains, veterinary source samples were processed at the Warrenton Regional Animal Health Laboratory. Those samples included 45 equine samples (nasopharyngeal and chest skin swabs from 19 mares and 6 nasal swabs from accompanying foals), nasopharyngeal and fecal samples from the original affected mare and her 1998 foal and placenta, and 32 environmental samples of stall
bedding, water and feed trough material, soil, and pasture, and fecal samples. Swabs from 9 employees were processed at a public health laboratory. All specimens were randomly coded at the time of culture, and results were decoded after reading of all 86 cultures.

All 32 environmental samples from the farm were negative for *C. diphtheriae* using a culture method that showed a 4-fold amplification of seeded bacterial numbers in a preliminary experiment. Environmental samples of stables and pastures and fecal samples were cultured as follows. Ten grams of sample material was added to 40 ml of serum tellurite blood broth in 50-ml sterile conical tubes, and samples were pre-incubated for 24 hours at 37 °C. After preincubation, the samples were vortexed thoroughly and centrifuged at 100 × g for 2 minutes to sediment large particulate matter. One hundred-microliter aliquots from each sample were then plated onto serum tellurite agar and Tinsdale medium and Columbia agar containing 5% sheep blood. Plates were incubated at 37 °C in 6% CO₂ for 24 hours prior to examination for the presence of coryneforme bacteria. Culture plates

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**Figure 1.** Growth characteristics of *Corynebacterium diphtheriae*. On Columbia agar plus 5% ovine blood, colonies of *C. diphtheriae* are gray to buff-tan. At 24–48 hours, the circular colonies are brittle and nonhemolytic. Upon staining with methylene blue, bacteria show characteristic metachromatic granules.

**Figure 2.** PCR assay used to detect the A and B subunits of the diphtheria toxin gene, *tox*. Lane 1, molecular weight marker; lane 2, *C. diphtheriae* strain A11; lane 3, *C. diphtheriae* strain A12 (duplicate of A11); lane 4, *C. diphtheriae* strain 510 (positive control); lane 5, *C. diphtheriae* strain 511 (negative control); lane 6, *C. diphtheriae* strain A11; lane 7, *C. diphtheriae* strain A12; lane 8, *C. diphtheriae* strain 510 (positive control); lane 9, *C. diphtheriae* strain 511 (negative control).
Figure 3. Elek immunoprecipitation assay used to determine the presence of the diphtheria toxin. P = toxigenic *C. diphtheriae* strain 510 (positive control); A11 = test strain; N = nontoxigenic *C. diphtheriae* strain 511 (negative control); A11 = test strain (duplicate); W = strain 3984 (weak positive control). Filter paper in the center of the plate contains antidiphtheria toxin antibody. Lines of precipitin formation between colonies and the filter indicates toxin production by the strain. Negative control strain has no precipitin line between the colony and the filter.

Figure 4. Ribotyping patterns of *C. diphtheriae* strain isolated from the affected mare (A11), 3 US isolates, and 3 Russian isolates (Lanes numbered left to right). Lane 1, strain A11 (*gravis* biotype, toxigenic); lane 2, *C. diphtheriae* strain E8392 South Dakota (*mitis* biotype, nontoxigenic); lane 3, *C. diphtheriae* strain G4218 Colorado (*gravis* biotype, toxigenic); lane 4, *C. diphtheriae* strain G4219 Alaska (*mitis* biotype, toxigenic); lane 5, strain B479 Russia (*mitis* biotype, toxigenic); lane 6, *C. diphtheriae* strain B480 Russia (*gravis* biotype, toxigenic); lane 7, *C. diphtheriae* strain B323 Russia (*mitis* biotype, toxigenic). Lanes 5–7 are predominant ribotypes observed during the recent Russian diphtheria epidemic.

were examined after 24 and 48 hours and 5 days of incubation. Water samples were filtered through 0.45-mm filters, and each filter membrane was incubated in a 5-ml aliquot of serum tellurite broth for 24 hours at 37°C prior to culture in identical manner as the other environmental samples. Nasopharyngeal, skin, and other swabs were plated directly onto Columbia agar/5% sheep’s blood and serum tellurite agar and Tinsdale medium in the same fashion as the environmental samples.

Toxigenic *C. diphtheriae* was not isolated in any of the samples collected on May 13. However, nontoxigenic *C. diphtheriae* was isolated from the placenta culture of the affected mare. In addition, *Streptococcus* strains were isolated from the uterine culture (*S. zooepidemicus*) and from the placenta culture and the draining wound (*S. equisimilis*). Contribution of *C. diphtheriae* to these mixed infections is of interest. In mixed infections, 1 bacterium can benefit from acquisition of metabolic by-products produced by the other types of bacteria. In the early 1900s, it was documented in Europe that *C. diphtheriae* aggravates strangles caused by *Streptococcus equi* in horses. In humans, simultaneous infection with *C. diphtheriae* and group A streptococci occurs. *Actinomyces* sp., which was isolated from the first wound culture, is also frequently found in mixed infections. Potentially, by-products associated with the *Streptococcus* spp. and the *Actinomyces* sp. may have allowed for extended colonization by *C. diphtheriae*.

Discovery of a wound infection associated with toxigenic *C. diphtheriae* from this animal reinforces our interest in horse as reservoirs for *C. diphtheriae*, facilitating its potential transmission to humans. However, no samples from equine or human contacts or from the surrounding environment yielded *C. diphtheriae*, so the mode of acquisition in this mare remains unknown.

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Assessment of the long-term shedding pattern of *Salmonella* serovar *choleraesuis* following experimental infection of neonatal piglets


**Abstract.** In the United States, swine salmonellosis is most often attributed to infections by *Salmonella* serovar *choleraesuis*. As a host-adapted pathogen rarely found in nonswine sources, *S. choleraesuis* is thought to be spread primarily via horizontal transmission, with carrier animals playing an important role. Little has been reported regarding infection of neonatal piglets, particularly regarding their potential to become carriers. Evidence reported herein demonstrates that piglets experimentally infected by *S. choleraesuis* at 2 days of age were capable of shedding the pathogen for up to 85 days postinfection, at which time the study was concluded. This study also presents findings supporting the use of GN-Hajna as a preenrichment medium for the isolation of *S. choleraesuis*.

*Salmonella choleraesuis* infections account for more than 90% of the US cases of swine salmonellosis.12 Salmonellosis caused by *S. choleraesuis* typically occurs as a postweaning septicemia or enterocolitis and often occurs in operations that commingle pigs of different ages.12 Spread of this host-adapted serotype throughout modern pig production systems is thought to occur primarily via horizontal transmission, which suggests an important role for asymptomatic carriers.5-7,12 *Salmonella choleraesuis* can recur in pigs previously infected, thus further implicating an important role for latent carriers.12 The development of a *S. choleraesuis* carrier state has been investigated experimentally with weaned piglets,5-7 but little is known regarding infection of neonatal piglets and their potential to shed the pathogen. Even though neonatal piglets can be infected, they rarely exhibit clinical salmonellosis.12 The primary objective of this study was to experimentally infect suckling piglets with *S. choleraesuis* and...

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