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Author(s):	**Vincent Fischetti, Ph.D.**
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Title Page

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Identification of Clostridium perfringens enterotoxin as a novel candidate trigger for Sudden Infant Death Syndrome

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Contact information of principal investigator:
Vincent Fischetti, PhD **(New Investigator)**
Professor and Head of the Laboratory of Bacterial Pathogenesis
The Rockefeller University
1230 York Avenue
New York, NY, 10065
Tel: 212-327-8166
Fax: 212-327-7584
Cell: 516-901-8400
Email: vaf@rockefeller.edu

Contact information of applicant organization:
Collette Ryder, MBA
Director, Sponsored Research and Program Development
The Rockefeller University
1230 York Avenue, Box 82
New York, NY, 10065-6399
Tel: 212 327 8054
Email: nihngas@rockefeller.edu

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SUMMARY OF STUDY FINDINGS

Under the auspices of the NIJ/DOJ, we have explored the hypothesis that sudden infant death syndrome (SIDS), may be triggered by environmental exposure to *Clostridium perfringens* enterotoxin (CPE). In this report, we show that the infant brainstem respiratory center is indeed susceptible to damage by bloodborne CPE dissemination, thus recapitulating the inflammatory scarring that has been observed in brain samples harvested from SIDS victims. In addition to brainstem expression of the CPE receptors, claudin 3 and claudin 4, we have also discovered brainstem expression of the rotavirus receptor, Junctional Adhesion Molecule 1 (JAM1), potentially implicating rotavirus infection as an alternate or coincident SIDS trigger. We have also characterized intestinal carriage of the *C. perfringens* bacterium and determined the prevalence of CPE gene carriage in fecal samples harvested from infants who have died suddenly; victims of sudden unexpected infant death (SUID). Of the 74 SUID fecal specimens collected, 26 samples (35%) were determined to be positive for *C. perfringens*. Furthermore, 16 of the 26 cultured *C. perfringens* strains (62%) carried the CPE gene. As commensal *C. perfringens* strains typically carry the CPE gene at a frequency of less than 15%, this four-fold CPE gene enrichment in SUID cases ($p = 0.0014$) strongly implicates CPE-producing *C. pefringens* strains in the pathogenesis of sudden infant death syndrome.

INTRODUCTION

Sudden Infant Death Syndrome (SIDS) is the leading cause of death in children less than one year of age (0.8/1000 live births).[1] Surely, the impact of SIDS on parents and families is tragic. However, the root cause of this disease remains unknown. Our current understanding of SIDS involves unexplained abnormalities in the respiratory center of the medulla oblongata (a portion of the brainstem); the thought being that SIDS victims have insufficient respiratory drive to adequately respond to episodes of apnea, where breathing ceases. The Ventral Medullary Surface (VMS) of the medulla oblongata, which controls respiratory drive, has come under investigation within the last few decades as neuropathologists have identified evidence of injury (astrogliosis) and neuronal loss within the arcuate nucleus of the VMS.[1,2,3] The neurons that comprise the arcuate nucleus/VMS express chemoreceptors that sense increasing levels of carbon dioxide in the blood, and activation of these neurons triggers the respiratory drive. The current theory is that selective neuronal loss and/or reduced neurotransmitter binding within the arcuate nucleus/VMS makes SIDS victims uniquely susceptible to accidental asphyxiation during periods of sleep.[1,2,3] Along these lines, having babies sleep in the supine position as opposed to the prone position has reduced the incidence of SIDS by thirty-eight percent.[3] Although a promising lead in SIDS research, how this region of the brain incurs damage still eludes us.

Bacterial toxins have also drawn attention in the pathogenesis of SIDS.[4-6] One toxin in particular, *Clostridium perfringens* enterotoxin, has been previously implicated.[7,8,9] Interestingly, investigators have found that *C. perfringens* can be identified in the intestinal tracts of up to 81% of SIDS infants compared to just 20% of healthy controls. Furthermore, damage to intestinal epithelium has been identified in 84% of SIDS cases and can be recapitulated in an animal model by exposing both mouse and rabbit intestine to enterotoxin.[8,9] Scanning electron microscopic comparison of the intestinal damage present in SIDS and experimental enterotoxin-mediated intestinal damage yields astonishingly similar pathologic images.[8] How enterotoxin might result in the lack of respiratory drive present in SIDS has been attributed to its effects as a "parasympathomimetic poison," which suppresses cardiorespiratory function[7,10,11], however, a definitive mechanism is yet to be identified.

Clostridium perfringens is a gram-positive bacillus, an obligate anaerobe, and importantly, a pathogen to humans in addition to domesticated and wild animals.[12-14] Like most Firmicutes, it is endospore forming and thus ever-present.[15-18] The species is typed based on production of one or more of 4 toxins: alpha, beta, epsilon, and iota[12-14, 19-22] (see Table 1). In addition to these toxins, each "toxinotype" is also capable of carrying the gene for *C. perfringens* enterotoxin (CPE), which is produced during the sporulation phase of the bacterium's life cycle.

Table 1. *Clostridium perfringens* toxinotypes, genotypes and associated diseases

Toxinotype	Toxin(s) encoded	Associated diseases
A	α	Humans: Gas gangrene Fowl: Necrotic enteritis. Foals, pigs: Diarrhea.
B	α, β, ε	Humans: Suspected in multiple sclerosis. Newborn Lambs: Dysentery. Newborn Calves and Foals: Hemorrhagic enteritis. Sheep and Goats: Enterotoxemia. Focal symmetric encephalomalacia.
C	α, β	Humans: Necrotic enteritis (Pigbel). Piglets, Lambs, Calves and Foals: Necrotic enteritis. Sheep: Enterotoxemia.
D	α, ε	Humans: Suspected in multiple sclerosis. Lambs, Sheep, Calves and Goats: Enterotoxemia. Focal symmetric encephalomalacia.
E	α, ι	Calves: Enterotoxemia.
F	α, CPE	Humans: Food poisoning, sporadic diarrhea, SIDS?

Adapted from Laetitia Petit et al. Trends in microbiology. 1999; Vol 7, Issue 3: 104-110

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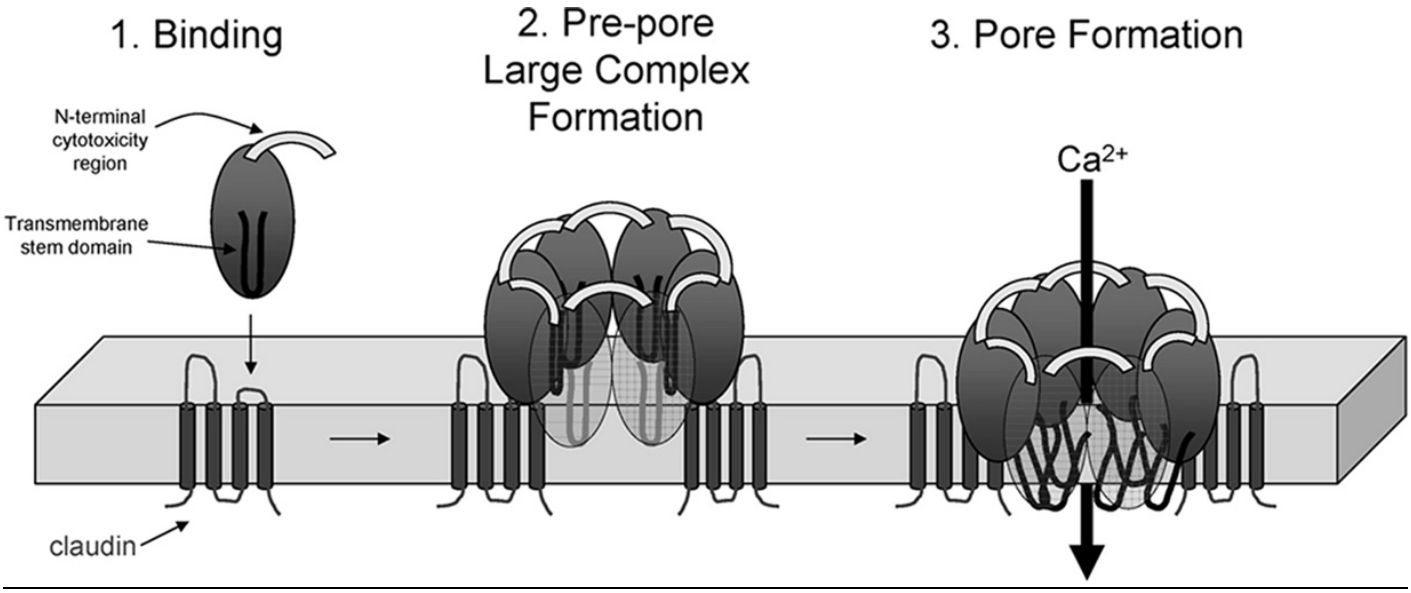
Alpha toxin is a chromosomally encoded phospholipase that cause cytolysis through destabilization of cell membranes.[12,14, 22-25] Beta toxin is a plasmid-encoded pore-forming toxin responsible for hemorrhagic necrosis of intestinal mucosa.[12,14, 22-25] Epsilon toxin is a plasmid-encoded pore-forming toxin responsible for CNS injury in goats and sheep characterized by blood-brain-barrier permeability, and focal, symmetric white matter disease.[12,14, 22-25] Iota toxin is a plasmid-encoded toxin with ADP-ribosylation activity specific for actin and resulting in cytoskeletal disruption.[16,22,25] Virulence for *C. perfringens*-encoded toxins depends on host factors such as species, age and body habitat, as well as bacterial factors and resident microbial communities that influence gene expression.

Enterotoxin is a 35 kilodalton exotoxin that is secreted during the sporulation phase of bacterium's life cycle. It is the third leading cause of food poisoning in adults and causes a mild gastroenteritis.[23,25] Interestingly, 20% of SIDS victims are reported to have suffered gastrointestinal symptoms within days leading up to sudden death. Another 66% have been found to have suffered upper respiratory infections, some of which with necrotizing features.[8] The cellular receptors for enterotoxin, i.e., tight junction proteins claudin-3 and claudin-4, were discovered at the turn of the century[26-29], and within recent years, other claudins have been found to bind enterotoxin but with binding affinities orders of magnitude lower than claudin-3 and claudin-4.[26,29] Typical for tight junction proteins, claudin-3 and claudin-4 are commonly expressed by barrier epithelium (e.g., enterocytes that form the intestinal lumen). As depicted in the schematic below, enterotoxin binds to claudin-expressing enterocytes and forms hexameric pores in the plasma membrane. Pore formation allows for an unregulated influx of calcium into the cell, resulting in cell death.[26,29]



James G. Smedley III et al. Infect. Immun. 2007;75:2381-2390

Preliminary Data

Although typically found in barrier epithelial cells, we have discovered neuronal expression of claudin-3 in a very restricted region of the rodent central nervous system. Indeed, only the medulla oblongata expresses claudin-3; specifically, the neural axons that comprise the VMS, the aforementioned respiratory center of the brain. CNS expression of the other major enterotoxin receptor, claudin-4, was not observed. This finding suggests that neurons that comprise the VMS are susceptible to enterotoxin-mediated damage. Furthermore, it may provide a mechanism for how enterotoxin might cause brain damage commonly found in SIDS victims on autopsy. The fact that the human blood-brain barrier (BBB) does not fully mature until 3-6 months of age, as evidenced by delayed multidrug resistant P-glycoprotein expression[30], suggests that there may be a window of time where small proteins such as enterotoxin may have access to the infant brain if present in the circulation. In addition, claudin-3 expression by BBB endothelial cells suggests that the BBB may become leaky if exposed to systemic enterotoxin.[31] Toxin access to brain tissue would surely damage the VMS and may unify the seemingly disparate brainstem vs. enterotoxin SIDS hypotheses. Identifying enterotoxin as a SIDS trigger would offer a unique opportunity for clinical intervention.

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RESULTS

Meningeal blood vessels of the medulla express CPE-receptors

For CPE to enter the infant brainstem, the toxin must first bypass the blood-brain barrier (BBB). As claudin proteins are integral components of the tight junction assemblies that form the BBB[32], we assessed claudin 3 expression within the rodent brain vasculature. Intriguingly, we observed claudin 3 expression by meningeal vessels underlying both the infant and adult ventral medullary surfaces (Fig. 1a and 1b). Outside of the ventral meningeal vessels, claudin 3 was expressed along the thin meningeal layer and in tight junctions of the choroid plexus (data not shown). However, claudin 3 was absent in meningeal vessels lining the dorsal brainstem (Fig 1c). Interestingly, we also observed exclusive expression of the high-affinity CPE receptor, claudin 4, by the meningeal vessels underlying the infant VMS (Fig. 1d). This is in contrast to what we had previously observed in the adult VMS, where claudin 4 was found to be absent. Neither claudin was expressed at an appreciable level by BBB vessels of the brain parenchyma proper (data not shown).

Fig 1.

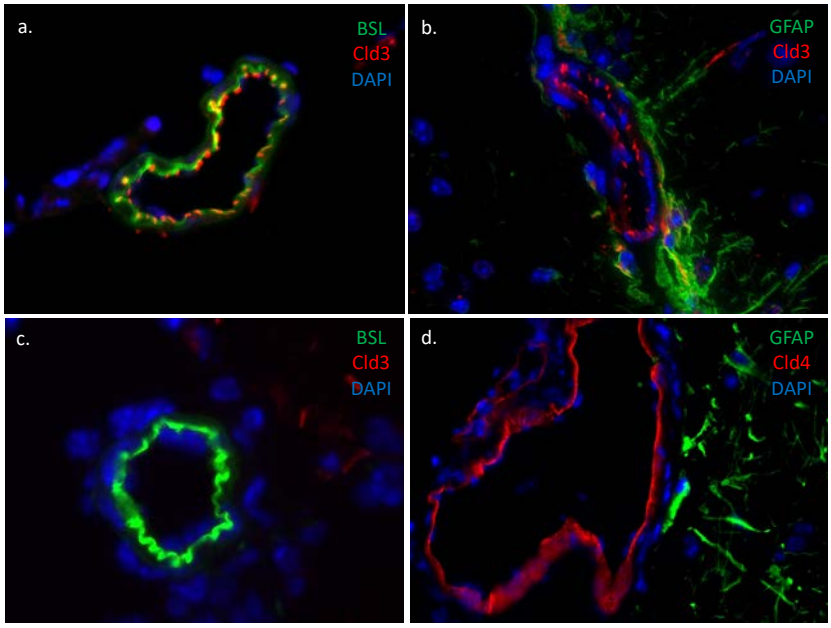

Figure 1. Meningeal vessels of the ventral medulla oblongata express claudin 3 and claudin 4
A) Sagittal brain section of an infant mouse (P11) showing a ventral meningeal vessel stained with BSL (a pan-vessel marker; green) and Cld3 (red). B) Coronal brain section of an adult mouse showing a ventral meningeal vessel stained with GFAP (astrocytes; green) and Cld3. C) Sagittal brain section of an infant mouse (P11) showing a dorsal meningeal vessel stained with BSL and Cld3. D) Sagittal brain section of an infant mouse (P11) showing a ventral meningeal vessel stained with GFAP and Cld4 (red). All images were taken at 40X magnification and DAPI staining visualizes cell nuclei.

Brainstem astrocytes express CPE receptor-claudin 3, and rotavirus receptor-JAM1.

As brainstem lesions in SIDS victims consist of reactive astrocytes, we sought to explore the possibility that CPE may directly target VMS astrocytes after bypassing the BBB. In support of this hypothesis, we observed claudin 3 expression by astrocytes forming the ventral brainstem glia limitans, which directly abuts the meningeal floor of both infant and adult brains (Fig. 2). In addition, to the VMS, we also observed claudin 3-positive astrocytes residing along the dorsal surface of the brainstem (data not shown). However, as previously stated, dorsal meningeal vessels failed to express claudin 3 (Fig. 1c) or claudin 4 (data not shown), rendering the dorsal medulla resistant to permeabilization by bloodborne CPE.

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Fig 2.

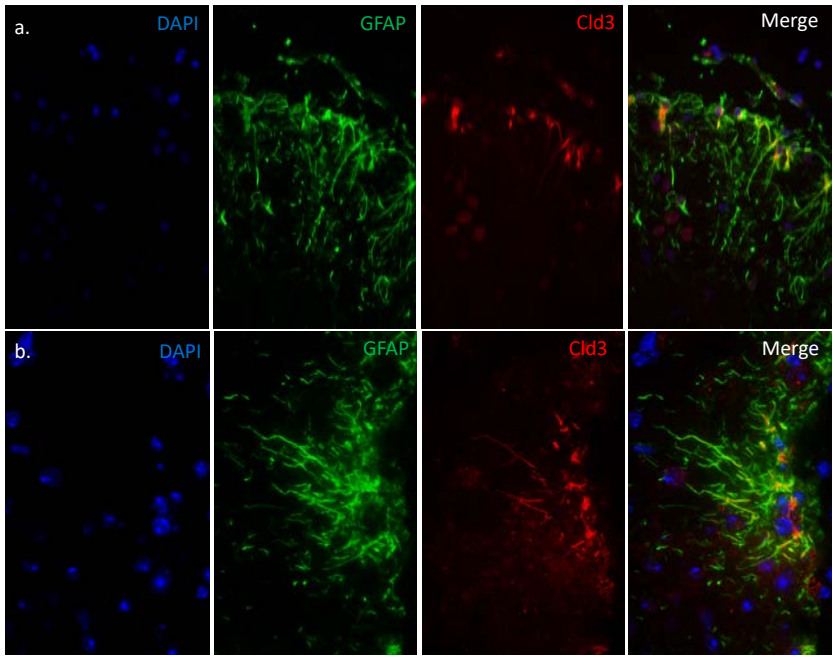

Figure 2. Brainstem astrocytes express claudin 3
A) Sagittal brain section of the infant mouse ventral medulla stained with GFAP (astrocytes; green) and claudin 3 (CPE receptor; red). B) Sagittal brain section of the adult mouse ventral medulla stained with GFAP and claudin 3. All images were taken at 40X magnification and DAPI staining visualizes cell nuclei.

Upon further characterization of astrocyte tight junction components, we unexpectedly observed rotavirus receptor-JAM1[33] expression by glia limitans astrocytes (Fig. 3). Unlike claudin 3, astrocyte JAM1 expression extended beyond the brainstem and could be observed in other regions of the brain glia limitans. Of note, intracellular tight junction adaptor proteins such as zonula occludens (e.g. ZO-1, ZO-2 and ZO-3) and homologous transmembrane Junctional Adhesion Molecules (JAM2 and JAM3) were all absent in brainstem astrocytes (data not shown).

Fig 3.

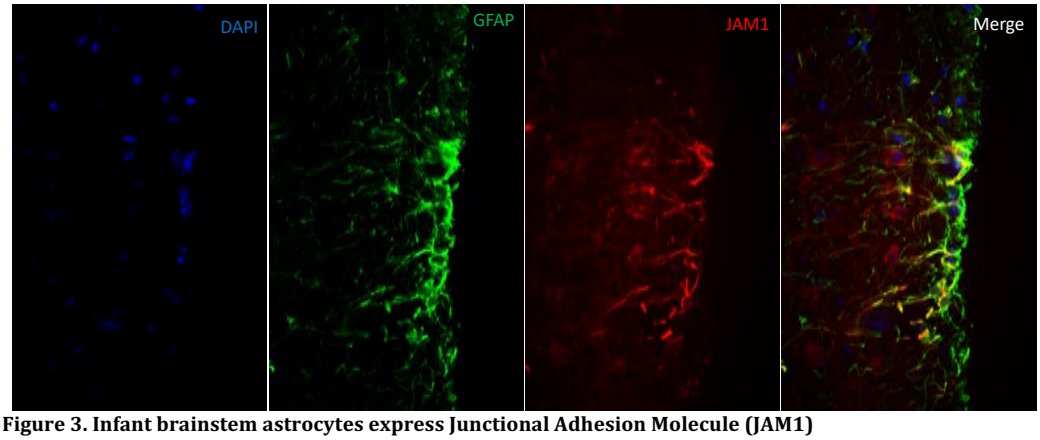

Figure 3. Infant brainstem astrocytes express Junctional Adhesion Molecule (JAM1)
A) Sagittal brain section of the infant mouse (P17) ventral medulla stained with GFAP (astrocytes; green) and JAM1 (rotavirus receptor; red). This image was taken at 40X magnification and DAPI staining visualizes cell nuclei.

Recombinant CPE binds to brainstem cranial nerves, but not to claudin 3 or claudin 4-positive brainstem structures
To determine the binding characteristics of CPE toxin in the rodent brainstem, we synthesized recombinant His-tagged CPE and stained tissues known to express claudin 3 and claudin 4, e.g. the kidney (Fig. 4) and the liver (not shown) as positive controls. The literature suggests that unexplained damaged to the thymus also occurs in SIDS cases[34], thus we wished to determine if recombinant CPE would bind to thymic tissue. We indeed observed CPE binding to thymic tissue sections (Fig. 4). Despite the expected staining patterns of kidney and liver tissue, we failed to observe appreciable CPE binding to the claudin 3 and claudin 4-positive blood vessels and astrocytes that we had identified in the ventral medulla. We instead observed robust staining of cranial nerve fascicles running along the ventral medulla (Fig. 5). We were unable to distinguish between cranial nerves IX, X, XI and XII, each of which emanate from the medulla.[35] However, it should be noted that cranial nerves IX, X and XII are each involved in respiration and/or airway patency.[35] As a negative control, we probed the same brain region using the

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same rabbit anti-CPE antibody, but with prior toxin incubation using a different recombinant His-tagged *C. perfringens* toxin, His-Epsilon toxin (His-ETX), to ensure that the anti-CPE signal was specific for recombinant CPE and not due to His-tag interference. Although, we did not observe specific His-ETX staining of the nerve fascicles, we did observe a relatively high level of non-specific background staining (Fig. 6).

Fig 4.

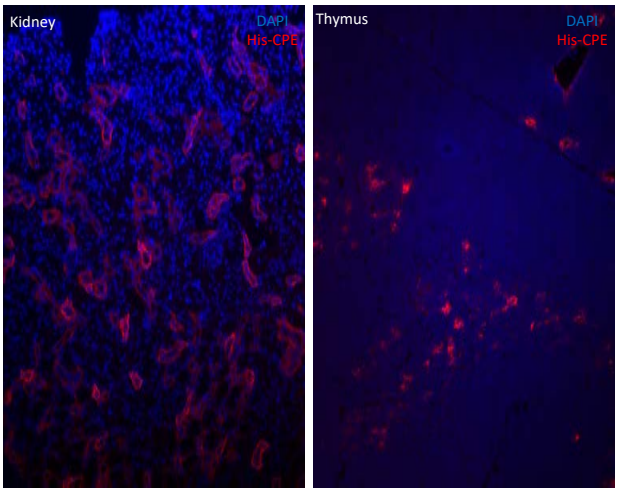

Figure 4. Recombinant His-tagged CPE stains kidney and thymus tissues
Left) Kidney, a known claudin-expressing tissue, stains positive for recombinant His-CPE (red); image taken at 20X.
Right) Thymus, a tissue reportedly damaged in SIDS, stains positive for recombinant His-CPE; image taken at 10X.
DAPI staining visualizes cell nuclei.

Fig 5.

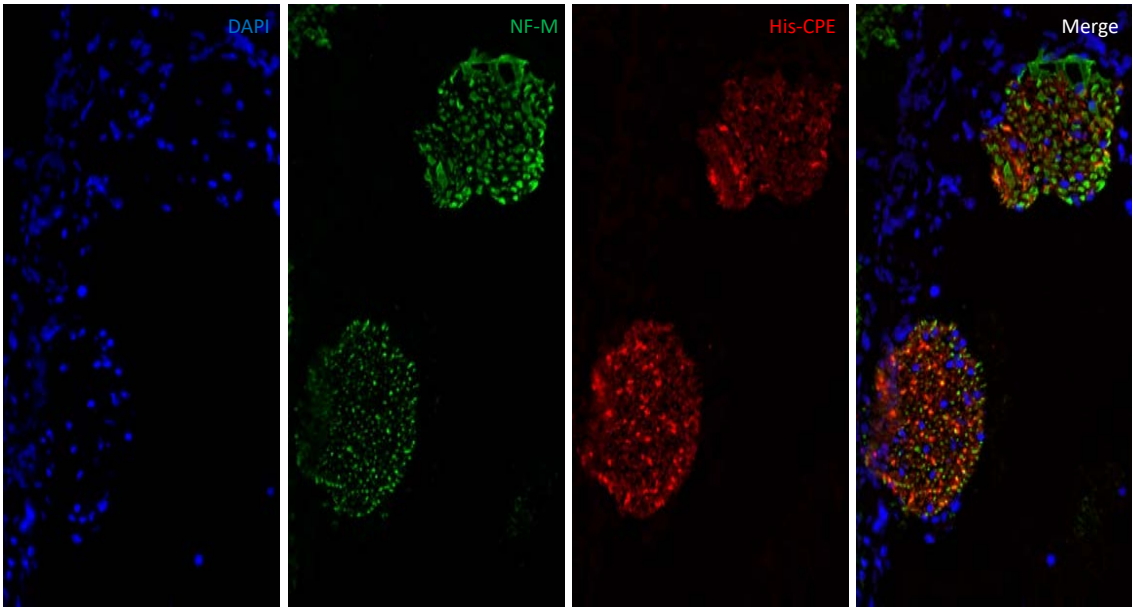

Figure 5. Recombinant His-CPE stains cranial nerve fascicles underlying the ventral medulla
Sagittal brain section of the infant (P11) ventral medulla co-labeled with neurofilament medium chain (NF-M; green) and His-tagged CPE (red). Image taken at 40X and DAPI staining visualizes cell nuclei.

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Fig 6.

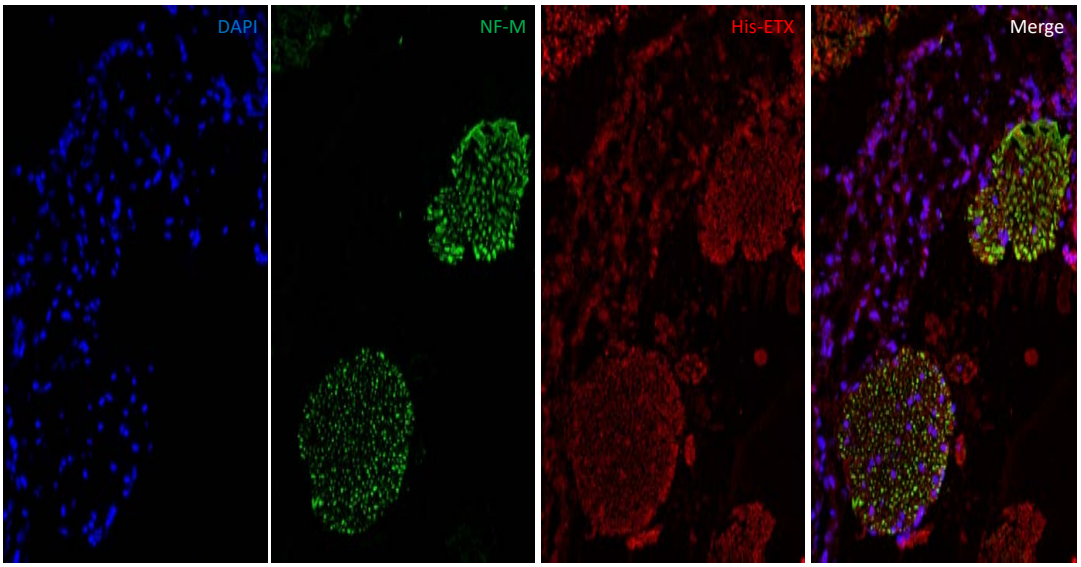

Figure 6. Recombinant His-ETX fails to stain cranial nerve fascicles underlying the ventral medulla
Sagittal brain section of the infant (P11) ventral medulla co-labeled with neurofilament medium chain (NF-M; green) and His-tagged ETX (red). Image taken at 40X and DAPI staining visualizes cell nuclei.

Systemic administration of native CPE causes brainstem inflammation.

Medullary astrogliosis (inflammatory scarring) and neuronal loss are commonly observed in SIDS victims.[1-3] In an effort to experimentally recapitulate these histological findings, we injected postnatal day 4, C57/B6 infant mice with 40ug/kg (half the LD50 dose)[36] of native CPE and allowed 7 days of recovery to allow for inflammatory brainstem changes to develop. After this 7-day period, we harvested brain tissue and assessed astrocyte expression of inducible nitric oxide synthase (iNOS), an enzyme that is expressed in the setting of acute inflammation.[37] While mice injected with an equal volume of sterile saline failed to display iNOS immunoreactivity (Fig. 7a), infant mice injected with 40ug/kg CPE displayed distinct regions of hypercellularity, which were populated by iNOS-positive reactive astrocytes (Fig. 7b). We next sought to assess if claudin 3 and JAM1-expressing astrocytes were significant constituents of these hypercellular clusters. Indeed, both claudin 3 and JAM1 antibodies successfully stained these regions of hypercellular astrocyte accumulations (Fig 8.)

Fig 7.

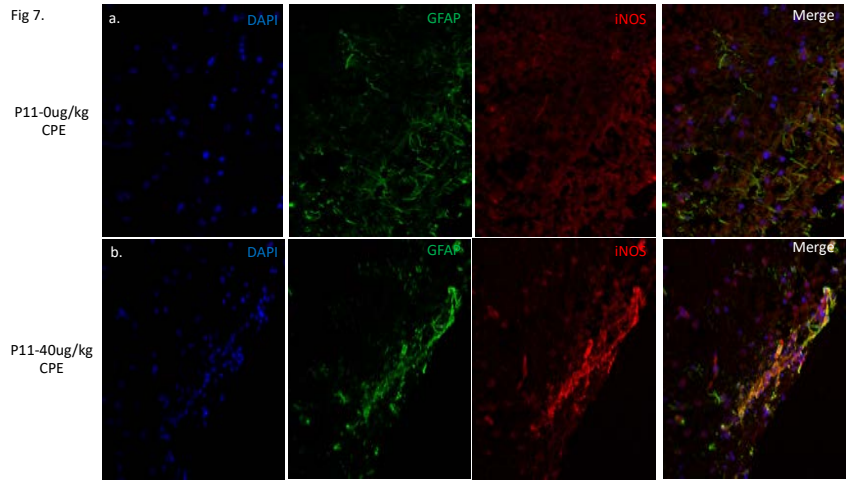

Figure 7. Systemic CPE administration causes hypercellular regions of iNOS-positive reactive astrocyte
A) Sagittal brain section of infant (P11) mouse ventral medulla, injected sterile saline (I.P.) 7 days prior to sacrifice. Astrocytes are labeled by GFAP antibody (green), and with an antibody against the inflammatory marker inducible nitric oxide (iNOS; red). B) Sagittal brain section of infant (P11) mouse ventral medulla, injected with 40ug/kg CPE (I.P.) 7 days prior to sacrifice. Astrocytes were stained for GFAP and co-labeled for iNOS immunoreactivity. All images were taken at 20X and DAPI staining visualizes cell nuclei.

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Fig 8.

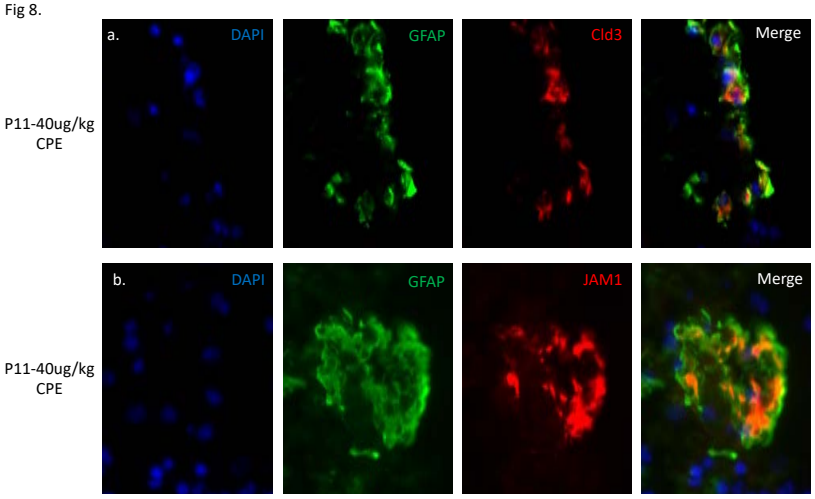

Figure 8. CPE-induced reactive astrocytes are both Cld3 and JAM1-positive
A) Sagittal brain section of infant (P11) mouse ventral medulla, injected with 40ug/kg CPE (I.P.) 7 days prior to sacrifice. Astrocytes were identified by staining for GFAP (green) and co-labeled with Cld3 staining (red). B) Sagittal brain section of infant (P11) mouse ventral medulla, injected with 40ug/kg CPE (I.P.) 7 days prior to sacrifice. Astrocytes were identified by staining for GFAP (green) and co-labeled with JAM1 staining (red). All images were taken at 40X and DAPI staining visualizes cell nuclei.

SUID fecal cultures yield an overabundance of CPE-carrying C. perfringens strains.
In coordination and collaboration with the Office of the Chief Medical Examiner (OCME) of New York City, we were able to collect fecal samples from 74 infants who died suddenly. Fifty-nine of these fecal samples were stored at room temperature prior to bacterial culture. While 15 of these samples were frozen prior to bacterial culture. Specimens were cultured in modified Rapid Perfringens Medium (RPM)[38], cultured bacteria were lysed, DNA was isolated and PCR was performed for 1) the 16s ribosomal RNA gene (16s), which is present in all bacteria; 2) *C. perfringens* alpha toxin, which is present in all *C. perfringens* strains; 3) the CPE gene, using the most commonly cited primer set[39], which yields a PCR product of 233 base pairs; and 4) CPE confirmation in the case of a CPE 233 base pair band that was either slightly above or, more often, slightly below the expected molecular weight. An authentic CPE signal was confirmed by using a different reverse primer[40] that would change the molecular weight of the CPE signal from 233 to 616 base pairs.

Specific diagnoses have been provided for 64 of the 74 samples by OCME thus far. Of the 74 fecal samples, 26 (35%) were determined to be positive for *C. perfringens* bacteria. Intriguingly, 16 of the 26 (62%) *C. perfringens* strains tested positive for the CPE gene ($p = 0.0014$, Fisher's exact test). When comparing CPE gene carriage to the designated cause of death, 12 of 16 (75%) were associated with SIDS; 3 of 16 (19%) were associated with a definable respiratory abnormality (1: interstitial pneumonia, 2: ileoceal intussusception complicated by upper respiratory infection, 3: compression of neck and chest), and 1 of 16 (6%) was associated with a non-respiratory cause of death (gunshot wounds of head). However, there were 25 of 74 cases (34%) identified as SIDS or death "undetermined" for which no CPE carriage could be readily identified (Table 2).

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TABLE 2. Fecal Culture PCR

Collection Date	Number	alpha toxin (324 base pairs)	CPE(233 base pairs)	CPE (616 base pair confirmation)	Observational Summary	OCME Diagnosis
2/9/18	1	†	†	N/A	C. perfringens (+); CPE-233 (+)	HYPOXIC-ISCHEMIC ENCEPHALOPATHY OF UNDETERMINED ETIOLOGY
2/16/18	2	–	–	N/A	C. perfringens (-)	INTERSTITIAL PNEUMONIA OF VIRAL ETIOLOGY(HUMAN RHINOVIRUS/ ENTEROVIRUS)
3/6/18	3	–	–	N/A	C. perfringens (-)	UNDETERMINED
3/26/18	4	†	†	N/A	C. perfringens (+); CPE-233 (+)	GUNSHOT WOUNDS OF HEAD
3/26/18	5	†	†	N/A	C. perfringens (+); CPE-233 (+)	COMPLICATIONS OF HYPOXIC-ISCHEMIC ENCEPHALOPATHY DUE TO SMOTHERING
4/3/18	6	†	†*	†	C. perfringens (+); CPE-233 (+*); CPE-616 (+)	ACUTE EXACERBATION OF CHRONIC BRONCHIAL ASTHMA. CONTRIBUTING CAUSE OF DEATH:INTERSTITIAL PNEUMONIA OF VIRAL ETIOLOGY (HUMAN RHINOVIRUS/ENTEROVIRUS
4/3/18	7	†	†*	–	C. perfringens (-)	BRONCHIOLITIS AND INTERSTITIAL PNEUMONIA OF VIRAL ETIOLOGY (HUMAN METAPNEUMOVIRUS AND RHINOVIRUS/ ENTEROVIRUS)
4/9/18	8	†	†*	†	C. perfringens (+); CPE-233 (+*); CPE-616 (+)	UNDETERMINED
4/23/18	9	†	†*	†	C. perfringens (+); CPE-233 (+*); CPE-616 (+)	ILEOCECAL INTUSSUSCEPTION. CONTRIBUTING CONDITION: UPPER RESPIRATORY INFECTION
5/7/18	10	†	†	N/A	C. perfringens (+); CPE-233 (+)	POSITIONAL ASPHYXIA
5/7/18	11	†	†	N/A	C. perfringens (+); CPE-233 (+)	UNDETERMINED (PRONE SLEEPING)
5/14/18	12	–	†*	N/A	C. perfringens (-)	SUDDEN INFANT DEATH SYNDROME
5/15/18	13	†	†*	†	C. perfringens (+); CPE-233 (+*); CPE-616 (+)	UNDETERMINED
5/15/18	14	–	–	N/A	C. perfringens (-)	COMPLICATIONS OFANOXIC ENCEPHALOPATHY OF UNDETERMINED ETIOLOGY
5/15/18	15	–	–	N/A	C. perfringens (-)	COMPLICATIONS OF BACTERIAL MENINGITIS (STREPTOCOCCUS PYOGENES)
6/6/18	16	†	†	N/A	C. perfringens (+); CPE-233 (+)	COMPRESSION OF NECK AND CHEST
6/15/18	17	–	†*	N/A	C. perfringens (-)	UNDETERMINED (PRONE SLEEPINGIN ADULT BED)
6/23/18	18	–	–	N/A	C. perfringens (-)	GROUP B STREPTOCOCCUS SEPSIS
7/5/18	19	–	†*	N/A	C. perfringens (-)	UNDETERMINED (PRONE SLEEPING POSITION AND RECENT/RESOLVING PARAINFLUENZA VIRAL INFECTION)
7/6/18	20	–	†*	N/A	C. perfringens (-)	CARDIAC ARRYTHMIAS DUE TO PROBABLE DSP GENE VARIENT NP_004406.2:P.PRO517LEU
7/12/18	21	†	†*	†	C. perfringens (+); CPE-233 (+*); CPE-616 (+)	UNDETERMINED (CO-SLEEPINGWITH ADULT ON ADULT BED)
7/13/18	22	†	–	N/A	**C. perfringens (+)**	Asphyxia due to Suffocation (Plastic Bag Covering Face)
7/19/18	23	–	–	N/A	C. perfringens (-)	COMPLICATIONS OF PREMATURITY
7/23/18	24	†	–	N/A	**C. perfringens (+)**	Undetermined (Bed-Sharing)
7/30/18	25	–	–	N/A	C. perfringens (-)	COMPLICATIONS OF CEREBRAL DYSGENESIS
8/1/18	26	–	–	N/A	C. perfringens (-)	DOWN SYNDROME WITH STATUS POST REPAIR OF TETRALOGY OF FALLOT WITH PROLONGED POST OPERATIVE RECOVERY DUE TO RESPIRATORY COMPROMISE COMPLICATED BY BRONCHOPNEUMONIA
8/9/18	27	–	†*	N/A	C. perfringens (-)	BACTERIAL BRONCHOPNEUMONIA COMPLICATING RESOLVING VIRAL BRONCHIOLITIS
8/27/18	28	–	–	N/A	C. perfringens (-)	INTRAUTERINE FETAL DEMISEDUE TO ABRUPTIO PLACENTAE DUE TO MATERNAL COCAINE USE
8/29/18	29	–	–	N/A	C. perfringens (-)	UNDETERMINED (CO-SLEEPINGIN ADULT BED)
9/13/18	30	–	–	N/A	C. perfringens (-)	BRONCHOPNEUMONIA COMPLICATING VIRAL UPPER RESPIRATORY TRACT INFECTION
9/17/18	31	–	–	N/A	C. perfringens (-)	SUDDEN UNEXPECTED DEATH IN INFANCY
9/17/18	32	–	†*	N/A	C. perfringens (-)	COMPLICATIONS OF UPPER RESPIRATORY INFECTION (LIKELY VIRAL) IN INFANT BORN PREMATURELY.
9/18/18	33	–	†*	N/A	C. perfringens (-)	HYPOXIC-ISCHEMIC ENCEPHALOMYELOPATHYOF UNDETERMINED ETIOLOGY
9/25/18	34	–	–	N/A	C. perfringens (-)	UNDETERMINED
10/22/18	35	–	†*	N/A	C. perfringens (-)	UNDETERMINED (CO-SLEEPING IN ADULT BED AND PROBABLEVIRAL TRACHEOBRONCHITIS)
10/29/18	36	†	†*	†	C. perfringens (+); CPE-233 (+*); CPE-616 (+)	UNDETERMINED (BED-SHARING)
10/29/18	37	†	†	N/A	C. perfringens (+); CPE-233 (+)	SUDDEN UNEXPECTED DEATH IN INFANCY
10/29/18	38	†	†*	†	C. perfringens (+); CPE-233 (+*); CPE-616 (+)	UNDETERMINED(PRONE SLEEPING POSITION IN ADULT BED)
11/2/18	39	†	–	N/A	**C. perfringens (+)**	UNDETERMINED(Bedsharing with adult)
11/8/18	40	†	–	N/A	**C. perfringens (+)**	UNDETERMINED(Sleeping on adult bed)
11/15/18	41	–	†*	N/A	C. perfringens (-)	UPPER AND LOWER RESPIRATORY TRACT INFECTION
11/27/18	42	†	†	N/A	C. perfringens (+); CPE-233 (+)	UNDETERMINED(SUDDEN INFANT DEATH; FOUND ON THE FLOOR AFTER BEDSHARING WITH AN ADULT)
12/3/18	43	–	†*	N/A	C. perfringens (-)	BRONCHOPULMONARY DYSPLASIA DUE TO PREMATURE BIRTH
12/13/18	44	–	†*	N/A	C. perfringens (-)	UNDETERMINED (BED-SHARING WITH ADULT ON ADULT BED WHILE SWADDLED)
7/26/19	45	–	–	N/A	C. perfringens (-)	pending further study
7/26/19	46	†	†	N/A	C. perfringens (+); CPE-233 (+)	UNDETERMINED
8/1/19	47	–	†*	N/A	C. perfringens (-)	ACUTE BACTERIAL PNEUMONIA
8/1/19	48	–	–	N/A	C. perfringens (-)	INFECTIOUS GASTROENTERITIS
8/2/19	49	–	–	N/A	C. perfringens (-)	UNDETERMINED
8/2/19	50	–	†*	N/A	C. perfringens (-)	MENINGOENCEPHALOMYELITIS, LIKELY VIRAL
8/4/19	51	–	–	N/A	C. perfringens (-)	pending further study
8/4/19	52	–	–	N/A	C. perfringens (-)	UNDETERMINED (COSLEEPING IN ADULT BED)
8/6/19	53 (freeze thawed)	–	–	N/A	C. perfringens (-)	POSITIONAL ASPHYXIA
8/6/19	54 (freeze thawed)	–	–	N/A	C. perfringens (-)	pending further study
8/11/19	55 (freeze thawed)	–	–	N/A	C. perfringens (-)	ACUTE MELATONIN INTOXICATION AND UNSAFE SLEEPING ENVIRONMENT
8/11/19	56 (freeze thawed)	–	–	N/A	C. perfringens (-)	UNDETERMINED, (BED SHARING WITH PARENTS)
8/12/19	57(freeze thawed)	–	–	N/A	C. perfringens (-)	SUDDEN UNEXPECTED DEATH IN INFANT WITH RECENT VIRAL SYNDROME (RESPIRATORY SYNCYTIAL VIRUS AND CORONAVIRUS)
8/12/19	58	–	–	N/A	C. perfringens (-)	UNDETERMINED (CO-SLEEPINGIN ADULT BEDWITH SOFT BEDDING)
8/15/19	59 (freeze thawed)	–	–	N/A	C. perfringens (-)	ASPHYXIA(FOUND FACE DOWN IN ADULT BED WHILE CO SLEEPING WITH ADULT)
8/15/19	60 (freeze thawed)	–	–	N/A	C. perfringens (-)	UNDETERMINED
8/17/19	61 (freeze thawed)	†	–	N/A	**C. perfringens (+)**	POSITIONAL ASPHYXIA (WEDGING OF BODY BETWEEN BED FURNITURE)
8/17/19	62 (freeze thawed)	–	–	N/A	C. perfringens (-)	UNDETERMINED (BED-SHARING WITH ADULT IN ADULT BED WITH SOFT BEDDING)
8/17/19	63 (freeze thawed)	†	–	N/A	**C. perfringens (+)**	ACUTE VIRAL (RHINOVIRUS/ENTEROVIRUS) BRONCHIOLITIS WITH HYPERSENSITIVITY REACTION
8/30/19	64 (freeze thawed)	–	–	N/A	C. perfringens (-)	POSITIONAL ASPHYXIA
9/1/19	65 (freeze thawed)	–	–	N/A	C. perfringens (-)	pending further study
9/6/19	66 (freeze thawed)	–	–	N/A	C. perfringens (-)	undetermined (four-day-old neonate in adult bed with adult and soft bedding)
9/13/19	67 (freeze thawed)	†	–	N/A	**C. perfringens (+)**	VIRALSYNDROME WITH MENINGOENCEPHALITIS ANDMYOCARDITIS
11/5/19	68 (freeze thawed)	†	–	N/A	**C. perfringens (+)**	pending further study
12/30/19	69	–	–	N/A	C. perfringens (-)	data pending
1/7/20	70	–	–	N/A	C. perfringens (-)	data pending
1/15/20	71	–	–	N/A	C. perfringens (-)	data pending
1/15/20	72	–	–	N/A	C. perfringens (-)	data pending
1/21/20	73	†	–	N/A	**C. perfringens (+)**	data pending
1/21/20	74	–	–	N/A	C. perfringens (-)	data pending

positive (+); suspected positive (+*); negative (-); CPE-positive after single PCR step; CPE confirmed by 2nd step PCR; non-respiratory illness

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DISCUSSION

In this study, we have revealed that the mammalian ventral brainstem expresses CPE receptor, claudin 3, in both the meningeal vessels that run along the ventral medullary floor and the astrocytes that comprise the adjoining glia limitans. Additionally, infant medullary meningeal vessels were found to also express the high-affinity CPE receptor, claudin 4, while adult vessels do not. Age-dependent expression of high-affinity CPE receptor claudin 4 may explain why SIDS only occurs in infants and not in mature adults. It is interesting to note that while medullary meningeal vessels displayed well defined, punctate or strand-like staining patterns (depending on the angle of the vessel within the tissue section), consistent with claudin 3 being a tight junction component, claudin 4 displayed a rather diffuse staining pattern with no clear evidence of tight junction incorporation.

In conflict with preliminary experiments performed prior to this study, we failed to observe claudin 3 expression by the axons that comprise the VMS neural network. Rather, we observed glial limitans astrocytes to be the predominant claudin 3 expressing cell type within the brain parenchyma. As the preliminary studies were conducted many years earlier, utilizing an antibody lot that is no longer available, we favor the idea that there may have been an unknown cross-reactivity issue with previous antibody lot that led to the recognition of neural axons. Alternatively, but less likely, different mouse strains may have different claudin 3 expression patterns within the brainstem. Furthermore, although mouse brainstem tissue expressed claudin 3 and claudin 4 in traditional places such as in blood vessels, and in non- traditional places, e.g., claudin 3 expression by medullary astrocytes, neither astrocytes nor medullary blood vessels stained positive when using recombinant CPE. This was surprising as recombinant CPE stained claudin-positive tissues such as the kidney and the liver. To clarify this discrepancy, although in preciously short supply, we will repeat these staining experiments using native CPE, produced by and purified from *C. perfringens* bacteria. From our work with other *C. perfringens* toxins e.g. Epsilon toxin, we are familiar with instances where toxin receptor-expressing tissues can go undetected by toxin staining and immunofluorescence detection. This is likely due to an insufficient level of receptor expression for detection via toxin incubation and anti-toxin antibody staining. In such cases, we have observed toxin-mediated cell death in the absence of appreciable histological staining, suggesting an imperceptible degree of toxin binding via histology, but successful toxin binding and biological activity.

While astrocytic claudin 3 expression fits nicely with the histopathological data of SIDS victims that identifies reactive astrogliosis as a hallmark of the SIDS brainstem lesion, neuronal loss within the arcuate nucleus requires further explanation. It has recently been shown by Liddelow et al. how reactive astrocytes (A1 toxic astrocytes) damage surrounding neurons.[41] Therefore, CPE's ability to target medullary astrocytes, thus transforming them into a reactive, toxic phenotype, may explain how surrounding arcuate nucleus neurons incur damage.

Surprising, we also identified astrocyte expression of another tight junction molecule, JAM1; the cellular receptor for rotavirus. As rotavirus is a pathogen that commonly affects infants, an optional vaccine is currently available.[42] Along these lines, we intend to cross-reference the rotavirus vaccination status of each infant in this study with the cause of death designation to explore the possibility that rotavirus susceptibility may be a contributing factor to SIDS via JAM1-expressing medullary astrocytes. Provocatively, a previous study has implicated rotavirus in SIDS, finding that 5 SIDS victims were positive for fecal carriage of rotavirus, compared to 0 of 36 non-SIDS infants, 6 of whom died of identifiable causes.[43] In addition to determining rotavirus vaccination status, we also plan to screen all of our collected fecal samples for the presence of rotavirus via a commercially available immunochromatography test. Future JAM1 immunohistochemical experiments will include a comparison of adult vs. infant JAM1 expression to determine if there is an age relationship to astrocyte JAM1 expression, and a possible age-related difference in brainstem susceptibility to rotavirus infection.

Our fecal culture results suggest a strong association between CPE exposure and SIDS, and perhaps also a relationship to death by respiratory compromise more generally e.g., respiratory infections. It was striking that of the 9 deaths caused by conditions unrelated to respiration, only one was positive for a CPE-producing *C. perfringens* strain. Beyond the present study, we envision extending our fecal culture experiments to healthy infants as live controls, so as to determine CPE-carriage rates for healthy infants under our unique experimental conditions. Without our own data set for healthy infants, we must rely on literature reports of > 15% carriage rates for commensal gut *C. perfringens* strains.[44] Finally, we observed a noticeable decrease in *C. perfringens* positivity when culturing fecal samples that had been frozen prior to bacterial culture. Despite this observation, we chose to include the frozen samples (15 in total) in our data to avoid the introduction of bias.

IMPLICATIONS FOR CRIMINAL JUSTICE PRACTICES

Although we have observed a four-fold enrichment of the CPE gene in strains harvested from SUID victims, and only 1 of 16 CPE-positive strains was associated with a non-respiratory cause of death (gunshot wound), we feel that our current assay is neither sensitive nor specific enough to be utilized for criminal justice determinations. Harvesting a CPE-positive strain from a victim should not be used as exculpatory evidence for wrong-doing, as the literature suggests that as many as 15% of healthy individuals can carry CPE-producing *C. perfringens* bacteria. Furthermore, the inability to register a CPE signal should not imply that SIDS has not occurred. In our study, there were 25 instances where SIDS cases were negative for CPE. Such a high false negative rate should discourage using the assay, in its current form, for criminal justice purposes.

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Major Project Phases as originally stated in grant application

Experiment	Start	End
1. Synthesis, purification and fluorescent labeling of enterotoxin (Aim 1)	01/01/17	06/30/17
2. Stain rodent brainstem with fluorescently labeled toxin (Aim 1)	07/01/17	9/30/17
3. Stain human infant brainstem with fluorescently labeled toxin (Aim 1)	07/01/17	12/31/17
4. Toxin administration to mice and assessment of brainstem injury (Aim 1)	07/01/17	06/30/18
5. Probe brainstems from SIDS victims for presence of enterotoxin (Aim 2)	07/01/17	12/31/19
6. Probe sera from SIDS victims for presence of enterotoxin (Aim 2)	07/01/17	12/31/19
7. Bacterial culture of SIDS intestinal contents and toxin gene PCR (Aim 2)	07/01/17	12/31/19
8. Characterization of SIDS microbiome by 16srRNA sequencing (Aim 3)	07/01/17	12/31/19

Please note that award was deferred by 6 months. Refer to updated project phase dates below:

Experiment	Start	End	% Complete	Completion Date	Status
1. Synthesis, purification and fluorescent labeling of enterotoxin (Aim 1)	07/01/17	12/31/17	100	10/3/17	complete
2. Stain rodent brainstem with fluorescently labeled toxin (Aim 1)	01/01/18	3/31/18	100	3/19/18	complete
3. Stain human infant brainstem with fluorescently labeled toxin (Aim 1)	01/01/18	06/30/18	0		abandoned
4. Toxin administration to mice and assessment of brainstem injury (Aim 1)	01/01/18	06/30/20	100	3/17/20	complete
5. Probe brainstems from SIDS victims for presence of enterotoxin (Aim 2)	01/01/18	06/30/20	0		abandoned
6. Probe sera from SIDS victims for presence of enterotoxin (Aim 2)	01/01/18	06/30/20	0		abandoned
7. Bacterial culture of SIDS intestinal contents and toxin gene PCR (Aim 2)	01/01/18	06/30/20	100	03/12/20	complete
8. Characterization of SIDS microbiome by 16srRNA sequencing (Aim 3)	01/01/18	06/30/20	0		abandoned

PARTICPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Vincent Fischetti PhD
Project Role:	Principal Investigator (Professor)
Nearest person month worked:	2
Contribution to Project:	Project Oversight/Supervision
Funding Support:	Salaried Faculty
Collaborated with individual in foreign country:	No
Country(ies) of foreign collaborator:	N/A
Travelled to foreign country:	N/A
If traveled to foreign country(ies), duration of stay:	N/A

Name:	Kareem Rashid Rumah MD, PhD
Project Role:	co-Principal Investigator (Postdoc)
Nearest person month worked:	12
Contribution to Project:	Project Oversight/Clinical Specimen Analysis/Animal handling/Histology
Funding Support:	N/A
Collaborated with individual in foreign country:	No
Country(ies) of foreign collaborator:	N/A
Travelled to foreign country:	N/A
If traveled to foreign country(ies), duration of stay:	N/A

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Name:	Olawale Eleso
Project Role:	Research Assistant
Nearest person month worked:	12
Contribution to Project:	Histology
Funding Support:	N/A
Collaborated with individual in foreign country:	No
Country(ies) of foreign collaborator:	N/A
Travelled to foreign country:	N/A
If traveled to foreign country(ies), duration of stay:	N/A

PARTNER ORGANIZATION

- Organization Name: Office of the Chief Medical Examiner (New York City)
- Location of Organization: 421 East 26th St, New York, NY 10016
- Partner's contribution to the project: Fecal Sample Harvesting
    - Financial support; Institutional
    - In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff); N/A
    - Facilities (e.g., project staff use the partner's facilities for project activities); N/A
    - Collaborative research (e.g., partner's staff work with project staff on the project); and
    - Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site). N/A
- More detail on partner and contribution (foreign or domestic). N/A

PRODUCTS/PUBLICATIONS/PRESENTATIONS

- Oral Presentation at The Rockefeller University Postdoc Retreat, September 19-20th 2019, Mohonk Mountain House, New Paltz NY

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