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Clostridium innocuum: Microbiological and Clinical Characteristics of a Potential Emerging Pathogen

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Abstract

Clostridium innocuum is an anaerobic, gram-positive, spore-forming bacterium identified by Smith and King in 1962 after being isolated from a patient with an appendiceal abscess. Its name, *C. innocuum*, reflected its clinically "innocuous" nature based on observed lack of virulence in animal models of infection. Since that time, *C. innocuum* has been identified as both part of the normal intestinal flora and the cause of a rare, intrinsically vancomycin-resistant opportunistic infection in immunocompromised patients. More recently, reports from Taiwan suggest that *C. innocuum*, in addition to being a known extraintestinal pathogen, may also be a diarrheal pathogen that causes a *C. difficile* infection-like antibiotic-associated diarrheal illness. However, unanswered questions about the clinical relevance of *C. innocuum* remain. Here we review the microbiological and clinical characteristics of this emerging pathogen.

INTRODUCTION

Clostridium innocuum was identified by Smith and King in 1962 [1] after being isolated from a patient with an appendiceal abscess. It was named *C. innocuum* as the researchers determined it was clinically "innocuous" based on an observed lack of virulence after intramuscular and intraperitoneal inoculation into guinea pigs and mice, respectively [1]. Since that time, *C. innocuum* has been identified as part of the normal intestinal flora [2] and a rare intrinsically vancomycin-resistant opportunistic infection in immunocompromised patients. More recent reports suggest *C. innocuum* pathogenicity may be overlooked [3, 4]. Here we review the microbiological and clinical characteristics of this potentially emerging pathogen.

CLINICAL MICROBIOLOGY

At the time *C. innocuum* was first reported, Smith and King characterized *C. innocuum* as an anaerobic, Gram-positive, spore-forming bacterium. The rods were 2-4 μm long by 0.4-1.0 μm wide, forming terminal oval spores. Colonies formed on agar were 1.5 to 2.5 mm in diameter, glossy, white, raised, with smooth margins. On blood agar plates, no hemolysis was observed. *C. innocuum* can ferment glucose, sucrose, salicin, and mannitol, but not

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lactose or sorbitol; maltose fermentation is slow and irregular. In regards to motility, *C. innocuum* was first described as non-motile [1]; however, recent genomic analysis of isolates suggested motility associated genes for type IV pili. This motility was confirmed with in vitro with agar-based motility assays [5]. These discrepancies further underscore the limited knowledge of *C. innocuum*.

Phylogeny

C. innocuum belongs to a metabolically diverse group, described as a clostriodia group, of low G+C Gram-positive eubacteria. Using 5S rRNA gene sequencing *C. innocuum*, along with *Clostridium ramosum*, are thought to be specific relatives to mycoplasma. This cluster of the mycoplasma group and two clostridia species originated as a deep phylogenetic branching in the Bacillus-Lactobacillus-Streptococcus branch of the Gram-positive evolutionary tree [6, 7].

Core genomic features of *C. innocuum* include lipid-derived metabolic substrates, multiple genes for lipid catabolism, and a functional substrate preference for β-hydroxybutyrate, a byproduct of fatty acid oxidation.

Laboratory Diagnosis

C. innocuum falls into the RIC group of *Clostridium* species (*C. ramosum*, *C. innocuum*, and *C. clostridioforme*) [8]. This group is routinely misidentified due to Gram-stain variability, rare formation of spores, atypical Clostridial colonial morphology, and variable antibiotic susceptibilities [8, 9]. Proper identification of *C. innocuum* with current laboratory microbiology methods and kits is difficult, and like its fellow group members, it is frequently misidentified [10]. Misidentification of *C. innocuum* as *Clostridioides difficile* has also been reported, as *C. innocuum* grows on cycloserine-cefoxitin-fructose agar (CCFA), a selective agar used for isolation of *C. difficile* from patients with diarrhea. Although *C. innocuum* grows readily on CCFA, and our personal experience in our laboratory supports this, the ability of *C. innocuum* to grow on other *C. difficile* selective media, such as chromogenic agar, is currently unknown. Exacerbating this misidentification is the similarity of both species' colony morphology [11]. Although the difference in odor of *C. innocuum* has not been reported, our personal experience in our laboratory suggests that like *C. difficile*, *C. innocuum* cultures typically demonstrate a horse barn odor, albeit not as strong as *C. difficile*.

While traditional phenotypic methods are capable of accurately identifying the genus of *C. innocuum*, these methods unreliably identify the species. Commercial anaerobic identification kits accurately identify *C. innocuum* 0-28% of the time [8]. Given the limitations of commercial anaerobic kits and phenotypic diagnostic methods, a more effective means of identifying *C. innocuum* is through molecular tools. Identification of *C. innocuum* has improved through analysis of metabolic products and fatty acids by gas-liquid chromatography (GLC), 16s rRNA gene sequencing, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) [8, 12]. Published literature suggests that commercial instruments such as Rapid ID32A and MALDI-TOF MS were unable to consistently and accurately identify clostridial species

outside of *C. perfringens* and *C. difficile*, demonstrating that limitations in accuracy still persist for some technologies [3]. However, identification of *C. innocuum* by commercial instruments in clinical microbiology laboratories by MALDI-TOF has recently improved. In the US, both the VITEK MS and Bruker biotyping systems have received clearance from the Food and Drug Administration for identifying *C. innocuum* from clinical samples with high confidence. Higher resolution and more genome sequence coverage may additionally be needed for the more accurate and rapid identification of *C. innocuum* from clinical samples using other technologies.

Antibiotic Resistance

C. innocuum is a rarely isolated Clostridial species by clinical microbiology laboratories that has displayed resistance to multiple antibiotics [13–19]. A summary of minimum inhibitory concentrations (MIC) for clinical isolates of *C. innocuum* compared to other commonly isolated Clostridia in the RIC group are listed in Table 1, adapted from Alexander et al. [8]. Clinical isolates were cultured from blood, intra-abdominal and soft tissue infections, and obtained from the R. M. Alden Culture Collection and from the Anaerobe Reference Unit. Resistance to several antibiotics, including cephalosporins, penicillin, and vancomycin has been demonstrated in *C. innocuum.* Although there are no specific interpretive standards for *C. innocuum*, Table 2 lists a summary of susceptibility breakpoints for other anaerobes per the Clinical and Laboratory Standards Institute (CLSI) [20] that have been previously applied to *C. innocuum* [3]. CLSI for vancomycin are not available for anaerobes, although for *C. difficile*, MIC ≥ 8 mg/L is considered resistant [20].

Intrinsic resistance to vancomycin is of particular concern. Although a prior study suggested the *C. innocuum* MIC90 for vancomycin is 8 mg/L,[8] a more recent study reported *C. innocuum* MIC90 to vancomycin was 16 mg/L; all 136 isolates in that study were resistant to vancomycin [4]. The genomic mechanism responsible for vancomycin resistance was first reported by David et al. in 2004 for *C. innocuum* NCIB 10674. The genes responsible are D-Alanine-D-alanine ligase, alanine racemase, and D-alanyl-D-alanine carboxypeptidase, which all encode for the synthesis of a peptidoglycan precursor with low vancomycin affinity [21]. *C. innocuum* resistance to multiple antimicrobials has led to challenges in management and treatment. However, these findings should be considered in the appropriate clinical context. For *C. difficile*, for example, the epidemiologic cut-off value for distinguishing isolates with elevated vancomycin MICs is ≥4 mg/L [22]. This cut-off value, which is similar to the *C. innocuum* MIC_{90} reported here (8-16 mg/L), is clinically relevant for systemic infections based on pharmacokinetics of intravenous vancomycin. However, enteral vancomycin, which is not well absorbed from the gut, can achieve colonic drug levels approximately 100-fold greater than the MIC_{90} of *C. innocuum* [23].

CLINICAL INFECTION

Extra-intestinal Clostridial infection

Since its discovery, *C. innocuum* has on rare occasions been reported to cause a variety of extra-intestinal Clostridial infections (EICI). Existing literature of *C. innocuum* extra-intestinal infection consists primarily of case reports or reports of rare *C. innocuum* infection

within larger reports of Clostridial infections in particular populations; the latter often lack clinical details about *C. innocuum* infections specifically. Table 3 summarizes several case reports of *C. innocuum* bacteremia, endocarditis, osteomyelitis, and peritonitis.

Gorbach et al. [24] described Clostridial infections in 114 patients; *C. innocuum* was reported to have caused 5 infections, including empyema (n=1), bacteremia (n=1), and soft tissue infection (n=3). Goldstein et al. [25] evaluated clinical isolates collected from post-operative infections in patients enrolled in a clinical trial comparing ertapenem and cefotetan for colorectal pre-operative prophylaxis. Among 104 patients with post-operative infections, *C. innocuum* was isolated from 13 patients, oftentimes as a polymicrobial infection. The site of infection and clinical significance of *C. innocuum* in these patients was not reported. Shah et al. [26], reported the clinical characteristics of Clostridial bloodstream infections at their center over an 8-year period; two patients had *C. innocuum* bacteremia, both of which were fatal [26]. Brook reported identification of Clostridia isolated from 96 children with various sites of infections; *C. innocuum* was isolated from 5 children, 4 with peritonitis and one with an intra-abdominal abscess [27].

Since publication of several case reports of rare opportunistic and other unusual presentations of *C. innocuum* infection, investigators in Taiwan have reported *C. innocuum* to be the second most common Clostridial species causing extra-intestinal infection. In a retrospective study conducted at the Chang Chung Memorial Hospital in Taiwan between 2007 and 2011, 375 non-repetitive Clostridial isolates from patients with EICI were collected from ascites, blood, and pleural fluid [3]. The isolates were identified using both phenotypic and genotypic methods. *C. perfringens* (190 cases) was the most common species identified in patients with EICI; *C. innocuum* (24 cases) and *C. difficile* and *Clostridium bifermentans* (18 cases each) were the next most common. Among these 24 patients with *C. innocuum* EICI, 19 had intra-abdominal infections and 5 had bacteremia. Associated symptoms/findings included diarrhea (8.3%), soft-tissue infections (12.5%), appendicitis (25%), shock (16.7%) and gastrointestinal perforation (16.7%). Notably, *C. innocuum* EICI was associated with a 16.7% mortality rate. The authors attempted to identify a potential mechanism through whole genome sequencing of the isolates; no known toxin genes were identified in *C. innocuum*.

Management and Treatment of Extra-intestinal *C. innocuum* infection

As described above, one of the greatest challenges of management and treatment of *C. innocuum* infection is its proper identification. Misidentification leads to inadequate treatment and complications for patients [8]. While there are no interpretive standards of antimicrobial susceptibility for *C. innocuum* specifically, Table 2 lists interpretive standards for anaerobes broadly.[20] Upon review of the literature presented here, the most commonly used antimicrobial agents were piperacillin-tazobactam, metronidazole, and clindamycin but comparative effectiveness studies are lacking.

Despite *C. innocuum* susceptibility to several antimicrobials, patient prognosis remains poor, with a mortality rate of 33.3% [28], likely related to the host co-morbidities associated with systemic infection. At this time there are currently no standardized treatment protocols. Thus, treating *C. innocuum* continues to pose challenges because of its common

misidentification, empiric use of vancomycin prior to identification and antimicrobial susceptibilities, and lack of standard treatment protocols for confirmed *C. innocuum*.

Antibiotic-Associated Diarrhea

In the last 20 years, several papers have been published in which *C. innocuum* was isolated from patients previously thought have CDI. In 2001, Ackermann and colleagues identified *C. innocuum* from the stool of three patients with recurrent *C. difficile*-associated diarrhea [11]. As previously mentioned, *C. innocuum* isolates can grow on CCFA, a selective agar used for isolation of *C. difficile* from patients with diarrhea. They were initially mischaracterized as *C. difficile* based on morphology. After the isolates were identified as negative for toxin A and B and vancomycin-resistant, further characterization by gas liquid chromatography (GLC) identified them as *C. innocuum.*

In another report, 48-year-old women was successfully treated with fidaxomicin followed by a tapering suppressive vancomycin regimen for CDI. After restarting vancomycin, she developed marked watery, yellow stools, similar to prior CDI episodes. Stool was negative for *C. difficile* by PCR and anaerobic culture. Upon further investigation *C. innocuum* was isolated from her stool and identified by MALDI-TOF [29]. The isolate was vancomycin-resistant (MIC: 48 ug/ml by E-test).

More convincing evidence of the role of *C. innocuum* in patients with diarrhea was recently reported by the same group of investigators from Taiwan who reported *C. innocuum* as in increasingly prevalent cause of EICI. They performed a retrospective study of patients with antibiotic-associated diarrhea (AAD) diagnosed between 2002-2007 [30]. From patients with AAD and thought to have CDI, they performed anaerobic stool culture using a *C. difficile*-selective agar, and isolates were identified by 16S rRNA sequencing. Among 2471 stool samples investigated, 136 (5.5%) were positive for *C. innocuum* by 16S rRNA PCR. Of these 136 samples, medical records were available from 103 patients. Watery diarrhea occurred in 63 (61.2%), bloody diarrhea in 28 (27.2%), mucoid diarrhea in ten (9.7%), and pseudomembranous colitis in two (1.9%). These symptoms were clinically indistinguishable from those of *C. difficile* infection, but colonies cultured from these stools were not identified as *C. difficile*. The patients were further classified into a severe colitis group and a diarrhea group based on clinical severity level. Severe colitis was defined as clinical presentation of either daily bowel output >1000 mL, presence of bloody stool, pseudomembranous colitis, or sepsis. Those that did not fulfill any of those requirements were placed in the diarrhea group. The overall mortality rate for the overall population was high (13.6%), and even higher (50%) in patients in the severe colitis group.

C. innocuum isolates were demonstrated to be predominantly resistant to vancomycin and susceptible to metronidazole, with minimum inhibitory concentrations of 90% of isolates to vancomycin and metronidazole to be at least 16 and 0.5 mg/L, respectively. These MICs are consistent with previously findings present in the literature (Table 1).

In vitro, these clinical isolates demonstrated cytotoxicity to Vero and HT-29 cells. In a mouse ileal loop model, *C. innocuum* isolates induced edema, inflammation, and necrosis, confirming intestinal pathogenicity of *C. innocuum* in an animal model. These findings

oppose the first characterization of *C. innocuum* as non-virulent in an animal model in the initial description of *C. innocuum* in 1962 [1]. In comparing these two assessments it's noted that pathogenicity was detected in a mice ileal loop model while, the first model failed to demonstrate pathogenicity in a non-intestinal model of infection. This suggests that *C. innocuum* mechanism of pathogenicity may be site specific.

Despite the intriguing findings from Chia et al. regarding *C. innocuum* pathogenicity in an animal model of infection, there were limitations in this study. The primary limitation was the lack of an asymptomatic control group to assess the prevalence of *C. innocuum* in the general population. Past investigation of *C. innocuum* in children with and without diarrhea resulted in similar rates of isolation, and the *C. innocuum* isolated were not cytotoxic to Vero cells [31]. Additionally, there was no proposed mechanism for *C. innocuum* pathogenicity. Because of the differing findings regarding virulence compared to initial assessments, further investigation is needed. To date, genomic analyses have confirmed that *C. innocuum* does not carry genes encoding toxins similar to that of *C. difficile tcdA* and *tcdB* [31]. Thus, the question remains as to the pathogenesis of the virulence observed by Chia et al.

PHYSIOLOGIC ROLE OF *C. INNOCUUM* IN THE GUT

As previously stated, *C. innocuum* has been characterized as a commensal gut microbe, an opportunistic pathogen, and the second most common Clostridial species responsible for EICI. At this point the potential of *C. innocuum* as pathogen remains largely unknown and current literature of *C. innocuum* varies greatly.

Most recently, Ha et al. [5] discovered an additional association of *C. innocuum* with gut health. New findings have implicated *C. innocuum* in tissue remodeling and inflammation in Crohn's Disease. *C. innocuum* was the mostly frequently isolated microbe from mesenteric adipose tissue from intestinal tissue inflamed by Crohn's disease. *C. innocuum* translocation to MAT and *ex-vivo* analysis show that it stimulates tissue remodeling via M2 macrophages promoting M2-like macrophages, increased microbial surveillance, wound-healing response, and adipogenesis [5]. These data suggest that *C. innocuum* may be acting in a protective manner in the gut. This assessment is in contrast to previous sections outlining the potential of *C. innocuum* as an emerging gastrointestinal pathogen causing antibiotic-associated diarrhea [3, 11, 29, 30].

Clinical and Clinical Microbiological Context of *C. innocuum*

There is still much to be learned about *C. innocuum* and its epidemiology, clinical significance, pathogenesis. Emerging data increasingly suggest that encountering *C. innocuum* in sterile sites should be considered representative of a true infection, rather than a contaminant or commensal, in the appropriate clinical context. Clinical microbiology laboratories that perform stool culture for *C. difficile* should be aware of the potential for misidentification of *C. innocuum* as *C. difficile* even when using selective media. Colony morphology, gram stain, and odor may not distinguish between the two organisms by culture. Clinical microbiology laboratories in the US using FDA-approved MALDI-TOF biotyper systems are able to accurately identify *C. innocuum* with high confidence.

CONCLUSION

Historical clinical experience suggests that *C. innocuum* is a relatively benign gut organism that causes rare, systemic opportunistic infection in immunocompromised and/or patients with comorbidities. However, recent reports from Taiwan suggest that *C. innocuum* may be an increasingly prevalent intrinsically vancomycin-resistant extraintestinal pathogen, as well as a diarrheal pathogen clinically indistinguishable from *C. difficile.* Despite these epidemiological findings, the pathogenesis of *C. innocuum* is largely unknown and requires further investigation.

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Table 1.

Susceptibility of *C. innocuum* and other clostridia to several antibiotics

	MIC_{90} (μg/ml)			
Antimicrobial Agent	***C. innocuum* (n=21)**	***C. perfringens* (n=11)**	***C. ramosum* (n=20)**	***C. clostridioforme* (n=20)**
Penicillin G	8	2	8	32
Metronidazole	1	2	1	0.125
Clindamycin	1	2	4	1
Cefoxitin	128	2	64	32
Cefotetan	>128	1	64	8
Imipenem	4	0.5	1	4
Meropenem	2	1	4	2
Amoxicillin-clavulanate	0.5	0.25	0.25	0.5
Ampicillin-sulbactam	0.5	0.25	1	2
Piperacillin-tazobactam	2	1	1	16
Vancomycin	8	0.25	2	0.25

Table adapted from [8]. Clinical isolates were cultured from blood, intra-abdominal and soft tissue infections, and obtained from the R. M. Alden Culture Collection and from the Anaerobe Reference Unit.

Table 2

C. innocuum antimicrobial susceptibility and resistance CLSI breakpoints

Antimicrobial	Susceptible	Resistant
Clindamycin	≤ 2 mg/L	≥ 8 mg/L
Metronidazole	≤ 8 mg/L	≥ 32 mg/L
Penicillin	≤ 0.5 mg/L	≥ 2 mg/L
Piperacillin-tazobactam	≤ 32/4 mg/L	≥ 128/4 mg/L
Ampicillin-sulbactam	≤ 8/4 mg/L	≥ 32/16 mg/L

Table adapted from [20]

Table 3.

Summary of case reports of *C. innocuum* infections

Author	Year	Patient characteristics	Infection Site	Antimicrobial resistance	Antibiotic Treatment (Antibiotic Duration)	Patient Outcome
Cutrona, et al. [32]	1995	18y F with no underlying medical conditions	Endocarditis	N/A	None; *C. innocuum* identified post-mortem	Died
Castiglioni, et al. [10]	2003	38y F with hepatitis C, kidney transplant, and chronic obstructive pulmonary disease	Bacteremia	vancomycin, cefoxitin	Piperacillin-tazobactam, penicillin G, clindamycin (11 days)	Recovered
Crum-Cianflone [33]	2009	38y M with acquired immunodeficiency syndrome	Bacteremia	vancomycin	metronidazole, linezolid (10 days)	Recovered
Hung, et al.[34]	2014	85y M with diabetes mellitus	Bacteremia	vancomycin	Piperacillin-tazobactam (2 weeks)	Recovered
Mutoh, et al.[28]	2015	32y M with acute lymphoblastic leukemia	Osteomyelitis and bacteremia	vancomycin	Piperacillin-tazobactam, metronidazole, clindamycin (11 weeks)	Recovered
Aroca-Ferri, et al.[35]	2019	44y F with takayasu's arteritis and chronic kidney disease	Peritonitis associated with peritoneal dialysis	vancomycin	cefotaxime, ampicillin, clindamycin (15 days until death)	Died

N/A- not applicable