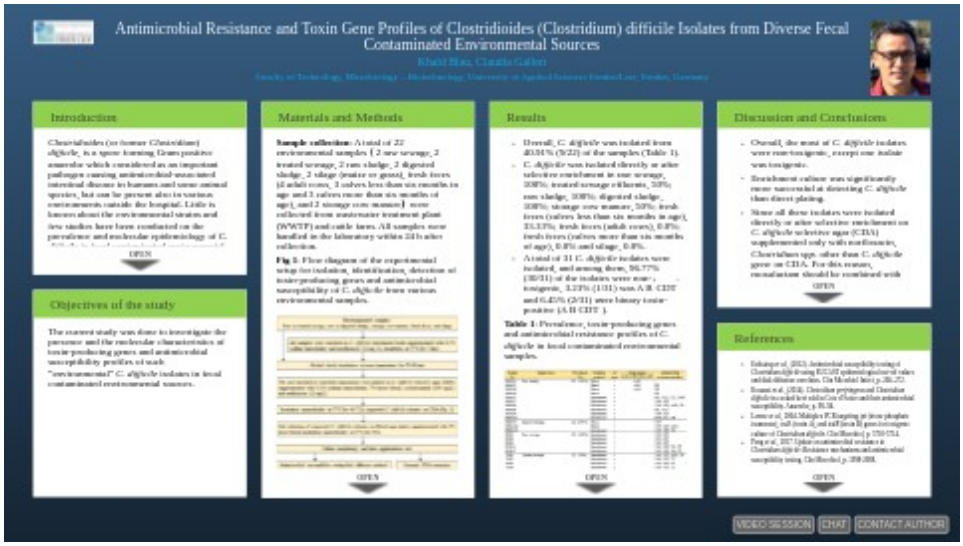
Antimicrobial Resistance and Toxin Gene Profiles of Clostridioides (Clostridium) difficile Isolates from Diverse Fecal Contaminated Environmental Sources



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INTRODUCTION

Clostridioides (or former *Clostridium*) *difficile*, is a spore forming Gram positive anaerobe which considered as an important pathogen causing antimicrobial-associated intestinal disease in humans and some animal species, but can be present also in various environments outside the hospital. Little is known about the environmental strains and few studies have been conducted on the prevalence and molecular epidemiology of *C. difficile* in fecal contaminated environmental sources. Therefore, optimization methods for isolation and molecular epidemiology of *C. difficile* are required to elucidate the role of the environmental sources as transmission routes for human infection.

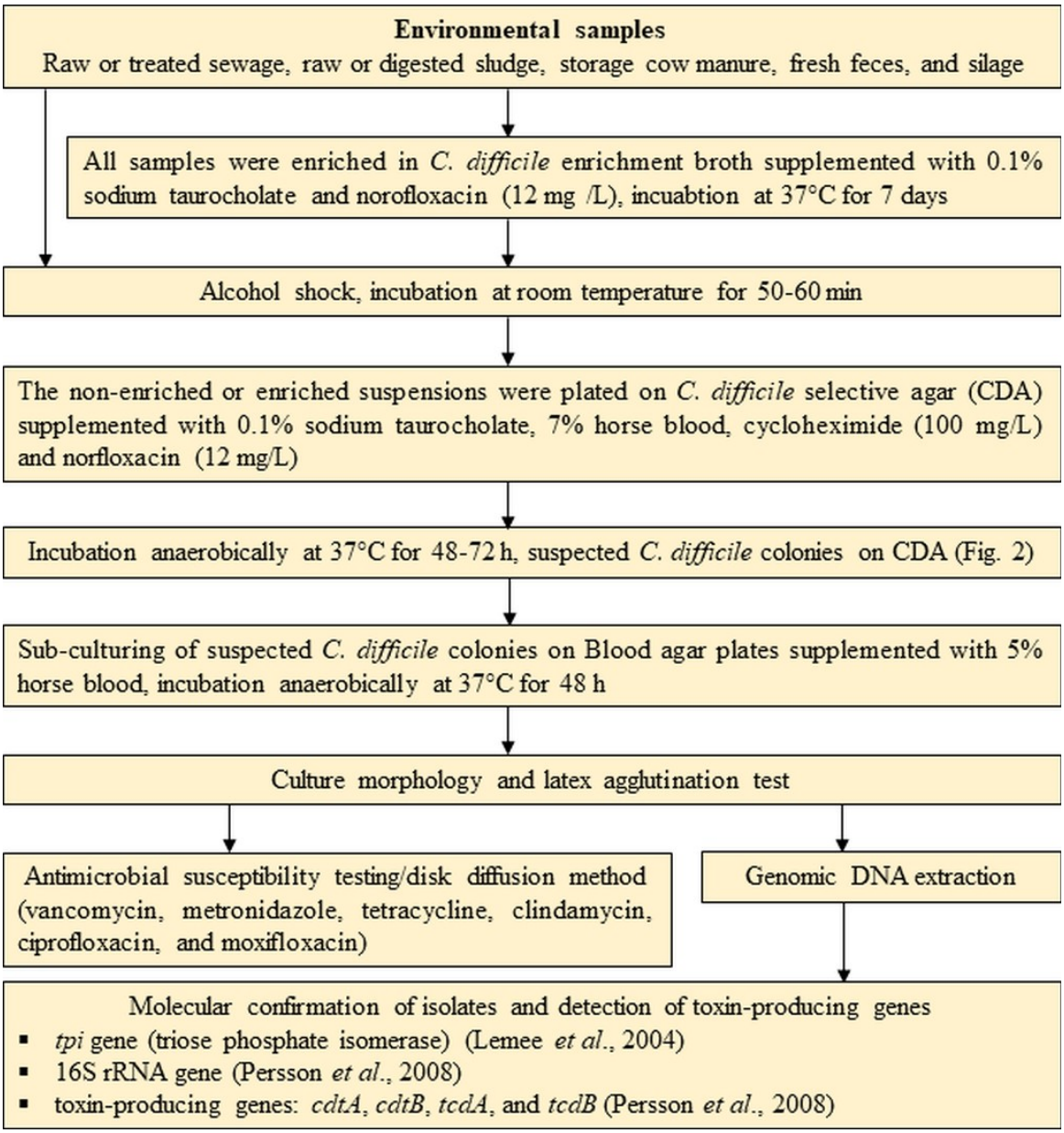
OBJECTIVES OF THE STUDY

The current study was done to investigate the presence and the molecular characteristics of toxin-producing genes and antimicrobial susceptibility profiles of such “environmental” *C. difficile* isolates in fecal contaminated environmental sources.

MATERIALS AND METHODS

Sample collection: A total of 22 environmental samples (2 raw sewage, 2 treated sewage, 2 raw sludge, 2 digested sludge, 2 silage (maize or grass), fresh feces (4 adult cows, 3 calves less than six months in age and 3 calves more than six months of age), and 2 storage cow manure) were collected from wastewater treatment plant (WWTP) and cattle farm. All samples were handled in the laboratory within 24 h after collection.

Fig 1: Flow diagram of the experimental setup for isolation, identification, detection of toxin-producing genes and antimicrobial susceptibility of *C. difficile* from various environmental samples.



RESULTS

- Overall, *C. difficile* was isolated from 40.91% (9/22) of the samples (Table 1).
- *C. difficile* was isolated directly or after selective enrichment in raw sewage, 100%; treated sewage effluents, 50%; raw sludge, 100%; digested sludge, 100%; storage cow manure, 50%; fresh feces (calves less than six months in age), 33.33%; fresh feces (adult cows), 0.0%; fresh feces (calves more than six months of age), 0.0% and silage, 0.0%.
- A total of 31 *C. difficile* isolates were isolated, and among them, 96.77% (30/31) of the isolates were non-toxigenic, 3.23% (1/31) was $A^-B^+CDT^-$ and 6.45% (2/31) were binary toxin-positive ($A^-B^-CDT^+$).

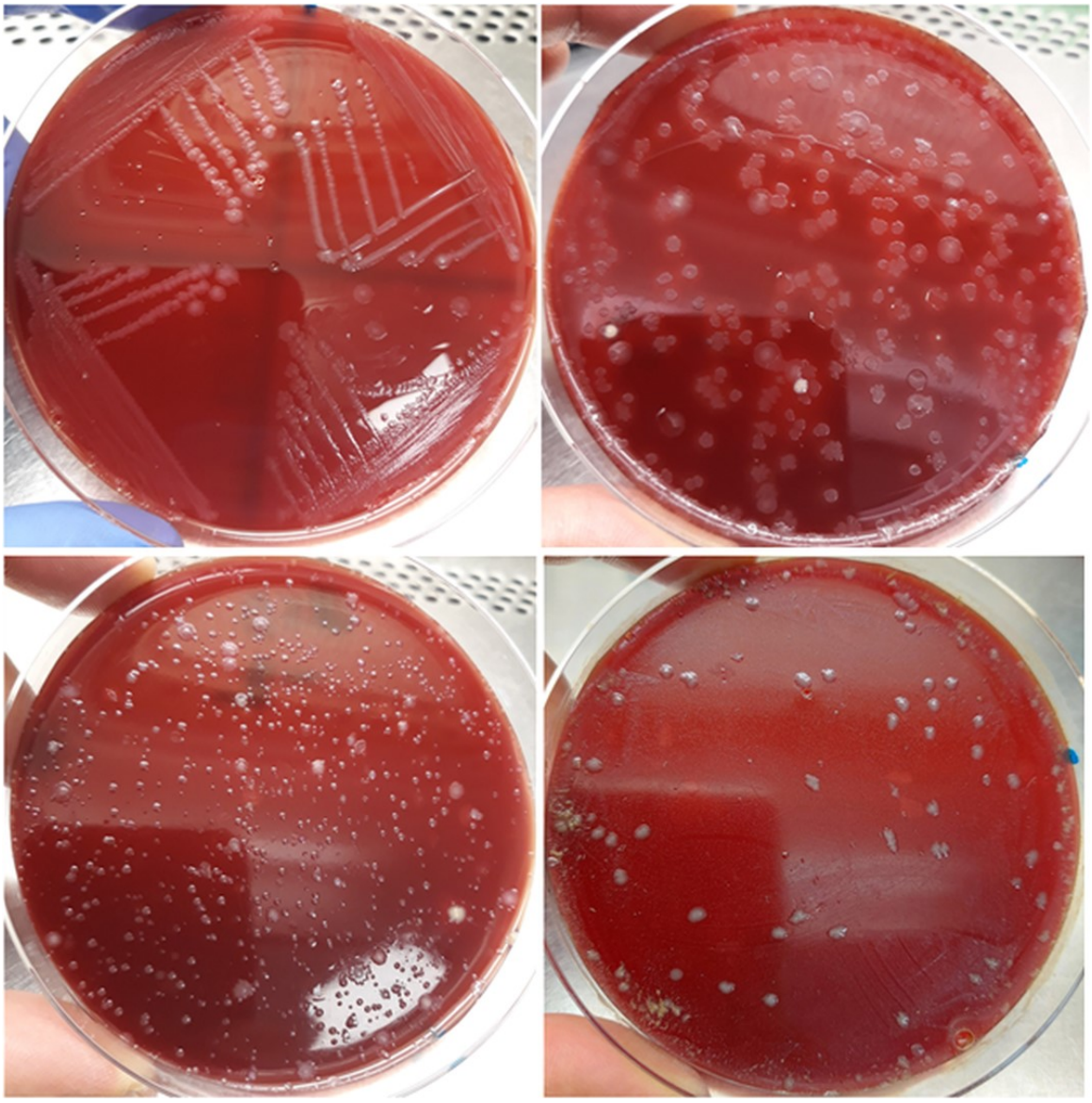
Table 1: Prevalence, toxin-producing genes and antimicrobial resistance profiles of *C. difficile* in fecal contaminated environmental samples.

Isolate No.	Sample type	Prevalence (%)	Isolation method	*tpi* gene	Toxin genes *tcdA*, *tcdB*, *cdtA*, *cdtB*	Antimicrobial resistance profiles
RSD-D4	Raw sludge	2/2 (100%)	Direct	+	*tcdB*	-
RSD-D7			Direct	+	*cdtB*	CIP
RSD-D8			Direct	+	*cdtB*	CIP
RSD-D9			Direct	+		CIP
RSD-E1			Enrichment	+		CIP, VAN, CLI, MXF
RSD-E2			Enrichment	+		VAN, CIP
RSD-E6			Enrichment	+		VAN, CIP, MXF, TE
RSD-E8			Enrichment	+		CIP, VAN
RSD-E9			Enrichment	+		VAN, CIP
RSD-E15			Enrichment	+		MTZ, CLI, CIP
DSD-D5	Digested sludge	2/2 (100%)	Direct	+		VAN
DSD-D22			Direct	+		VAN, CIP, MTZ
DSD-E10			Enrichment	+		VAN, CIP, CLI
RS-E1	Raw sewage	2/2 (100%)	Enrichment	+		VAN, CIP
RS-E2			Enrichment	+		VAN, CIP
RS-E4			Enrichment	+		VAN, CIP
RS-E11			Enrichment	+		VAN, CIP, CLI, TE
RS-E15			Enrichment	+		VAN, CIP, CLI, TE
TS-E1	Treated sewage	1/2 (50%)	Enrichment	+		VAN, CIP, MXF, TE
TS-E2			Enrichment	+		VAN, CIP, TE
TS-E3			Enrichment	+		VAN, CIP, TE
TS-E4			Enrichment	+		VAN, CIP, TE
TS-E5			Enrichment	+		VAN, CIP, TE
TS-E6			Enrichment	+		VAN, CIP, TE
TS-E7			Enrichment	+		VAN, CIP, TE
TS-E8			Enrichment	+		VAN, CIP, TE
TS-E10			Enrichment	+		VAN, CIP, TE
CD3.1.1D	Fresh feces (calves less than six months age)	1/3 (33.3%)	Direct	+		MTZ, CIP, VAN
CD3.1.2D			Direct	+		MTZ, CIP, VAN
CD3.1.3D			Direct	+		MTZ, CIP, VAN
SM1-D	Storage cow manure	1/2 (50%)	Direct	+		MTZ, CIP, VAN
nd.	Silage (grass or maize)	0/2 (0.0%)				
nd.	Fresh feces (calves more than six months age)	0/3 (0.0%)				
nd.	Fresh feces (adult cows)	0/3 (0.0%)				

VAN: vancomycin, CIP: ciprofloxacin, TE: tetracycline, CLI: clindamycin, MXF: moxifloxacin, MTZ: metronidazole. nd: not detected. Interpretation of inhibition zone: breakpoints of VAN, MXF and MTZ (Erikstrup *et al.*, 2012); Breakponits of TE and CIP (Kouassi *et al.*, 2014); CLI breakpoint as recommended by members of the SFM Antibiogram Committee (2020).

- One toxigenic isolate ($A^-B^+CDT^-$) was susceptible to all tested antimicrobials; while most non-toxigenic isolates were resistant to more than two antimicrobials of different classes. The resistance against fluoroquinolones (ciprofloxacin) is very common (93.55% of the isolates tested), glycopeptide antimicrobials (vancomycin, 83.87%), and tetracyclines (tetracycline, 38.71%), followed by lincosamide (clindamycin) and metronidazole (nitroimidazoles).

Fig. 2: Suspected *C. difficile* colonies (grey-white and irregular) on CDA plates.



DISCUSSION AND CONCLUSIONS

- Overall, the most of *C. difficile* isolates were non-toxigenic, except one isolate was toxigenic.
- Enrichment culture was significantly more successful at detecting *C. difficile* than direct plating.
- Since all these isolates were isolated directly or after selective enrichment on *C. difficile* selective agar (CDA) supplemented only with norfloxacin, *Clostridium spp.* other than *C. difficile* grew on CDA. For this reason, moxalactam should be combined with norfloxacin at concentration 32 mg/L as this inhibited the growth of *Clostridium spp.* (other than *C. difficile*).
- The *C. difficile* was found in fresh feces of calves less than six months in age, but not in other fecal samples (adult cows and calves more than six months of age), which considered to be a major reservoir of *C. difficile.*
- The majority of non-toxigenic isolates were found to be resistant to ciprofloxacin, vancomycin and tetracycline. These isolates could be carried tetracycline resistance genes [such as *tet*(M) and *tet*(W)] and macrolide-lincosamide-streptogramin B (MLS_B) resistance genes (*ermB*) often located on the mobile genetic elements (MGEs) such as conjugative transposons (Peng *et al*., 2017).
- *C. difficile* strains might be acquired resistance to tetracycline and clindamycin via the transfer of MGEs among *C. difficile* strains and/or between *C. difficile* and the other bacterial species, especially conjugative transposons (Tn*5397* and Tn*916* which associated with tetracycline resistance, *tet*(M) and Tn*5398*, Tn*6194* and Tn*6215* associated with MLS_B family, ermB (Peng *et al*., 2017).
- *C. difficile* strains are commonly present in various fecal contaminated environmental sources, which could be serve as a potential source of community-associated *C. difficile* infection.
- Molecular epidemiology is needed to determine the clinical relevance of environmental *C. difficile* strains isolated from environmental sources.

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