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Molecular epidemiology and antimicrobial resistance of *Clostridioides difficile* detected in chicken, soil and human samples from Zimbabwe

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ABSTRACT

Background: Clostridioides difficile is the major cause of infectious nosocomial diarrhoea in industrialized nations. Data on the occurrence of *C. difficile* in Africa, ribotype (RT) distribution, antimicrobial susceptibility patterns and potential zoonotic transmission are scarce.
Methods: 80 Zimbabwean *C. difficile* isolates from different sources (chicken [$n = 30$], soil [$n = 21$] and humans [$n = 29$]) were investigated using ribotyping, toxin gene detection, resistance testing, multiple-locus variable-number tandem repeat analysis (MLVA), and whole genome sequencing (WGS).
Results: Among chicken isolates, the most common RTs were RT103 (6/30), RT025 (5/30) and RT070 (4/30). Within soil samples, RT025 and RT056 were most common (3/21 each). In contrast, the non-toxigenic RT084 was most frequently found in human isolates (4/29). Toxin genes were detected in only 19/29 human isolates. Susceptibility testing showed no resistance against metronidazole and vancomycin, and resistance against macrolides and rifampicin was scarce (3/80 and 2/80, respectively); however, 26/80 isolates showed moxifloxacin resistance. MLVA and WGS of strains with identical RTs stemming from different sources revealed clustering of RT025 and RT084 isolates from human und non-human samples.
Conclusion: No "hypervirulent" strains were found. The detected clusters between human, chicken and soil isolates indicate ongoing transmission between humans and environmental sources and might point towards a zoonotic potential.

Introduction

Clostridioides difficile (formerly *Clostridium difficile*) is a Gram-positive, anaerobic, rod-shaped bacterium and the main cause of nosocomial diarrhoea in industrialized nations, thus posing a considerable burden on the healthcare system. Its ability to form spores facilitates its spread within the environment (e.g. in the hospital setting) (Weber et al., 2013). Most strains obtained from clinical infections produce two distinct toxins (toxin A and B, encoded by *tcdA* and *tcdB*, respectively), while a third toxin, i.e. the binary toxin, encoded by *cdtAB*, is associated with more virulent isolates (Gerding et al., 2014). Non-toxigenic isolates can be considered apathogenic and might hold a protective effect towards colonization with toxigenic strains (Natarajan et al., 2013). The carrier rate in the healthy human population ranges from 0% to 15% according to studies that were, however, predominately conducted in industrialized nations (Furuya-Kanamori et al., 2015), while prevalence in infants may be even ≥80% (Matsuki et al., 2005). *C. difficile* is ubiquitously found in nature and can be isolated from

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environmental sources (e.g. food, soil and water) and from a broad variety of different animal species, including livestock (e.g. poultry, swine, and cattle) (al Saif and Brazier, 1996; Moono et al., 2017; Kotila et al., 2013; Hensgens et al., 2012). At the beginning of the 21st century, human *C. difficile* epidemiology has been shaped by the introduction of "hypervirulent" strains such as ribotype (RT) 027. This "hypervirulent" strain emerged from North America and spread to a number of other regions of the world, especially to South America and Europe, during the last two decades (He et al., 2013). In Europe, RT027 constitutes nowadays up to 19% of *C. difficile* isolates obtained from infections (Davies et al., 2016). However, although this RT has caused sporadic cases and outbreaks in other parts of the world [e.g. East Asia and Australia (Richards et al., 2011; Cheng et al., 2016)], RT027 has not yet been isolated in Africa.

It is important to note that the RT distribution differs significantly between different continents: In East Asia, RT017 is frequently detected, which is rarely found in other regions of the world. In contrast, in North America, Europe, Australia and other parts of Asia, RTs such as RT001, RT014 and RT027 are prevalent strains (Davies et al., 2016; Knight et al., 2015; Collins et al., 2013; Tickler et al., 2014; Davies et al., 2014).

In Africa, however, *C. difficile* infection (CDI) is a largely neglected disease, and epidemiological data on the pathogen are scarce (Becker et al., 2015). In African HIV patients, *C. difficile* was suggested as a possible neglected pathogen (Seugendo et al., 2015), and was discussed in African children to be of clinical relevance (Plants-Paris et al., 2019). The most accurate molecular data available to date originate from South Africa, where a high prevalence of RT017 was noted (Samie et al., 2008; Kullin et al., 2017, 2018).

More recently, the zoonotic potential of *C. difficile* was acknowledged (Hensgens et al., 2012), and several possible zoonotic RTs, such as RT078, were identified from both human and animal sources (Goorhuis et al., 2008a,b; Alvarez-Perez et al., 2013; Schneeberg et al., 2013). In subsequent studies, a broad variety of livestock including pigs (Alvarez-Perez et al., 2013), bovines (Magistrali et al., 2015), and poultry (Harvey et al., 2011) such as pigeons (Abdel-Glil et al., 2018), chicken (Razmyar et al., 2017), quails (Zamani et al., 2019), ducks and swallows (Bandelj et al., 2014) were found to be colonized with *C. difficile*. Common RTs being frequently encountered in human disease and poultry are RT001 (Abdel-Glil et al., 2018; Indra et al., 2009), RT002 (Hussain et al., 2016), RT014 (Hussain et al., 2016), RT027 (Varshney et al., 2014), RT039 (Abdel-Glil et al., 2018), and RT078 (Varshney et al., 2014; Weese et al., 2010), supporting a potential role of poultry in zoonotic *C. difficile* transmission.

In this study, we determined the molecular subtypes and antibiotic susceptibilities of 80 *C. difficile* isolates from human, poultry (chicken), and soil samples that mainly stemmed from two studies conducted in Zimbabwe (Simango and Mwakurudza, 2008; Simango and Uladi, 2014) to elucidate their epidemiology and zoonotic potential.

Materials and methods

Study populations and sample collection

Overall, 80 *C. difficile* isolates were included in the study: 30 isolates stemmed mainly from chicken faeces and 21 isolates from soil samples, which were mainly collected from different market places in the capital city of Harare in North Central Zimbabwe in 2005 (Simango and Mwakurudza, 2008), and 29 human isolates stemming from 29 outpatients who were treated at several healthcare facilities in Harare in 2008. Among these 29 isolates 23 toxigenic strains were reported (Simango and Uladi, 2014).

Laboratory testing

Isolates were cultured and identified as described previously (Simango and Mwakurudza, 2008), using standard biochemical and morphological identification methods (culture morphology and latex agglutination) in Zimbabwe as described previously (Simango and Mwakurudza, 2008; Simango and Uladi, 2014). For further characterization, strains were sent to the German National Reference Center (NRZ) for *C. difficile*. Anaerobic culture, ribotyping, toxin gene detection, and antibiotic susceptibility testing (AST) was carried out as described previously (Berger et al., 2018, 2019a). Anaerobic culture was done on a selective agar (CLO-Agar, bioMérieux; Marcy L'Étoile, France). Ribotyping and toxin gene detection were performed in accordance with harmonized protocols (ECDIS-net), and AST was done by epsilometry for metronidazole, vancomycin and moxifloxacin, and by disk diffusion for clarithromycin and rifampicin.

To determine the epidemiological relationship among samples, all isolates displaying the same RT were subtyped using multiple-locus variable-number tandem repeat analysis (MLVA) as described before (Berger et al., 2019a; Färber et al., 2017). Clonality was defined as $\leq$2 repeat differences and relatedness as $\leq$10 differences (Berger et al., 2019a; Färber et al., 2017). Since the study focused in part on zoonotic transmission, whole genome sequencing (WGS) was applied on samples that clustered in MLVA and included human and non-human samples in the same cluster. Moreover, two unclassified strains with the binary toxin gene were whole genome sequenced. Allelic profiles for subsequent core genome MLST (cgMLST) analysis were extracted from the sequence data as described previously (Illumina Inc., San Diego, USA) (Bletz et al., 2018; Berger et al., 2019b) with one modification. We used the SKESA assembler (Souvorov et al., 2018) for *de novo* assembly with default parameters. Isolates differing in $\leq$6 alleles were considered to cluster.

Results

In total, 80 isolates were investigated that originated from 30 chicken samples, 21 soil samples, and 29 human specimens (Table 1). Overall, a high diversity of RTs with a total of 35 different RTs was observed, including 15 yet unclassified RTs which did not match with the available RT profiles in the institutional database of the German NRZ for *C. difficile*.

The RT distribution displayed a partial source specificity: In chicken isolates, RT103 [6/30, (20%)], RT025 [5/30 (17%)] and RT070 [4/30, (13%)] were the most common RTs, while RT025 and RT056 were most prevalent in soil samples [3/21 each (14%) Table 1]. In human samples, RT084 was most frequently detected with 4/29 (14%), followed by RT002, RT012, RT046, and RT056 [2/29, (7%), each, Table 1]. Classical "hypervirulent" RTs such as RT027 or RT078 were absent in this strain set. However, the binary toxin gene *cdtAB* was detected in two strains, representing yet unclassified RTs corresponding to the multilocus sequence types (ST) 122 and ST664, respectively.

A strikingly high number of toxigenic isolates was found in chicken and soil samples [26/30, (87%) and 20/21, (95%) respectively], while only 19/29 (65%) human samples were positive for *tcdA* and/or *tcdB*.

MLVA was carried out for all RTs, which were represented by a least two isolates encountered more than once, showing varying degrees of relatedness (17 RTs, data not shown). Of these, eight RTs (isolates) did not show any clustering. In the remaining nine RTs, a total of ten clusters could be detected. In four cases, isolates obtained from chicken and soil samples clustered, in two cases each, either only human or only chicken samples clustered together. The remaining two clusters included human- and non-human-derived

Table 1
List of detected ribotypes (RTs) of the 80 isolates investigated. Isolates clustering in MLVA: C: chicken, S: soil, H: human. Each letter symbolises one isolate (e.g. in RT025, a total of 8 samples were tested: 5 chicken, 3 soil and 1 human specimen; two clusters were found: one cluster with two samples both from chicken and one cluster with four samples, two from chicken, one from soil and one human). None: no samples clustered, n.a. not applicable since the RT was detected only once. "Unclassified RTs" did not match with RTs of our database (17 isolates). Eleven of these were different RTs. Four isolates had of two different banding patterns [RT (A) and RT (B)].

RT	Chicken ($n=30$)	Soil ($n=21$)	Human ($n=29$)	Isolates in a MLVA cluster
Number of strains carrying toxin genes *tcdA* and *tcdB*				
RT001	1	1	1	CS
RT002	–	–	2	None
RT005	–	–	1	n.a.
RT012	–	–	2	HH
RT014	2	2	1	CS
RT017[a]	–	–	1	n.a.
RT020	–	1	1	None
RT025	5	3	1	CC; CSH
RT046	–	–	2	None
RT053	1	1	–	None
RT056	–	3	2	None
RT070	4	1	–	CCS
RT103	6	–	1	CCCCC
RT120	2	1	–	None
RT031	1	0	0	n.a.
RT207	2	1	–	CS
RT220	1	–	–	n.a.
RT228	1	1	1	None
Unclassified RTs	1	4[b]	2[b]	n.a.
Unclassified RT (A)	–	1	1	None
Strains carrying no toxin genes				
RT031	1	–	–	n.a.
RT084	2	–	4	CHHHH
Unclassified RTs	1	1	4	n.a.
Unclassified RT (B)	–	–	2	HH

[a] RT017 isolates frequently harbour mutations in *cdtA*, and might not be identified correctly by certain PCR-based diagnostic methods, and toxin A is normally not expressed (Du et al., 2014).
[b] Additional two *cdtAB* positive isolates in the soil and human sample set

isolates: one RT025 cluster comprised isolates originating from human, chicken and soils samples (Supplementary Figure 1), and the RT084 cluster included a couple of samples stemming from human and chicken samples (Supplementary Figure 2).

WGS and subsequent cgMLST was applied in RT025 and RT084 isolates since human and non-human samples clustered in MLVA (Figure 1 and Supplementary Figures 1 and 2). The results of WGS (Figure 1) and MLVA (Table 1 and Supplementary Figures 1 and 2) were similar but not completely congruent in two out of three clusters. Using WGS (ENA accession number: PRJEB36842), one isolate did not cluster in contrast to MLVA (RT025; isolate 35C, Figure 1), while in the other cluster, one sample was additionally related in WGS (RT084; isolate 26C, Figure 1).

Resistance against drugs used for therapy of CDI in Europe and North America such as vancomycin and metronidazole was not detected in any sample (Table 2). However, resistance towards moxifloxacin was high in chicken, soil, and human samples [11/30 (37%), 9/21 (43%) and 6/29 (21%), respectively]. Rifampicin and clarithromycin resistances were occasionally detected in human- and chicken-derived isolates (<10%), but not in soil isolates. Detailed resistance patterns of individual isolates are displayed in Supplementary Table 1.

Discussion

Data regarding the prevalence and molecular epidemiology of *C. difficile* in Africa are scarce, and its impact on local healthcare systems remains unclear. Some studies, however, point toward a possible significance in HIV-positive patients (Seugendo et al., 2015; Onwueme et al., 2011). In one study conducted in Kenya, a high rate of toxigenic *C. difficile* (92%) was noted in stools received from diarrheal patients with HIV (Oyaro et al., 2018). In Zimbabwe, the clinical significance of this pathogen has not been consistently acknowledged (CDDEP, 2017), although in 9% of diarrheal outpatients, toxigenic *C. difficile* could be identified in one study (Simango and Uladi, 2014).

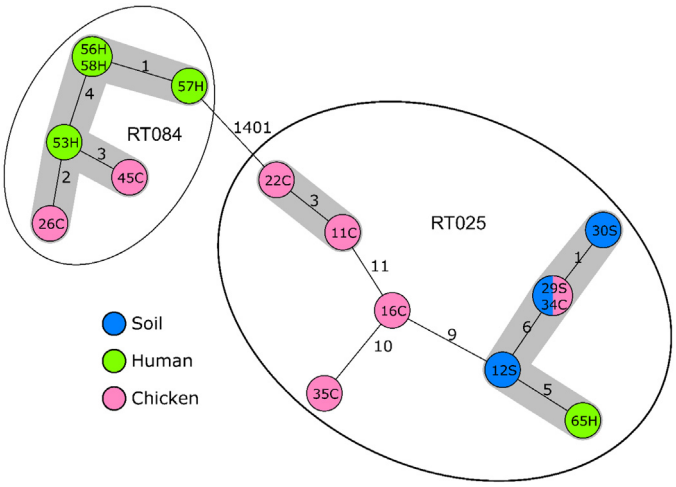

Figure 1. Minimum-spanning tree of 17 *C. difficile* isolates from different origins. Each node represents a unique cgMLST allele profile and named with the sample ID. The numbers on connecting lines display the number of differing alleles between the genotypes (line length not to scale). The different nodes are colored by the sample's origin and closely related genotypes (≤6 different cgMLST alleles) are shaded with grey.

The emergence and spread of "hypervirulent" RTs such as RT027 and RT078 altered the epidemiological *C. difficile* world map, and this change was particularly evident for North America and Europe, leading to a higher burden of *C. difficile* disease in humans in those regions (Cairns et al., 2017). An active surveillance including monitoring of these more virulent RT is therefore crucial in order to take countermeasures (e.g. antibiotic stewardship, implementation of hygiene measures) when the respective RT is introduced. Therefore, the assessment of the RT composition is also important for identification of regions that can be considered vulnerable.

In this study, a large RT diversity was observed among human isolates. Interestingly, a comparably high rate of non-toxigenic isolates was noted within the human-derived isolate set [10/29 (34%)]. Human RTs reported to be highly prevalent in other world regions such as RT001, RT002, RT014, or RT017 were only rarely found [in total 5/29 (17%)]. Importantly, no "hypervirulent" RTs such as RT027 and RT078 were present. However, in one human-derived isolate, the binary toxin gene could be detected, which is commonly found in isolates of these "hypervirulent" RTs, and is considered to be associated with a more severe course of clinical disease (Gerding et al., 2014).

These findings are largely in line with those of other studies conducted thus far in African patients (Supplementary Table 2), with the exception of South Africa. In samples from South African patients with diarrhoea, the rate of non-toxigenic *C. difficile* was below 10% in two studies (Samie et al., 2008; Kullin et al., 2018). Additionally, in South Africa, RT017 is by far the most common RT found in humans. Within an outpatient setting, RT017 and RT001 made up 50% and 16% of all isolates, respectively (Samie et al., 2008). In South African tuberculosis hospitals, RT017 was even found in 96% and 64% of all RTs, respectively (Kullin et al., 2017, 2018). Zimbabwe and South Africa share a common border. Therefore, it might be speculated that the exchange of *C. difficile* reservoirs (e.g. through trade and migration) might influence and

Table 2
Detected resistance rates among all three sample sets (chicken, soil and humans).

Sample set	Metronidazole	Vancomycin	Moxifloxacin	Clarithromycin	Rifampicin
Chicken	0/30	0/30	11/30	1/30	0/30
Soil	0/21	0/21	9/21	0/21	0/21
Human	0/29	0/29	6/29	2/29	2/29
All	0/80	0/80	26/80	3/80	2/80

shape the local epidemiology. However, our findings do not support this hypothesis, as the RT compositions differ largely between our study and the data from South Africa. RT017 was found only once in our strain set, and only few RTs observed in the study presented here were also detected in the aforementioned South African studies (RT001, RT002, RT014, and RT046, respectively) (Samie et al., 2008; Kullin et al., 2017, 2018).

For most parts of Africa, molecular data on *C. difficile* are scarce. In an Algerian study, eleven isolates were tested of which four were non-toxigenic RT084 (Djebbar et al., 2018). According to an investigation from Côte d'Ivoire (Becker et al., 2015) only 2/6 isolates could be assigned to a certain RT (RT199 and RT390, respectively). Of note, all of the reported Côte d'Ivoire isolates were non-toxigenic (Becker et al., 2015). A study from Ghana identified only three toxigenic isolates within a total of fifteen isolates (Janssen et al., 2016). An investigation conducted in Tanzania reported three RT038 (non-toxigenic), two RT045 (toxigenic), and two unknown toxigenic RTs (Seugendo et al., 2015). Of note, a high incidence for RT084 and a comparatively high proportion of non-toxigenic strains were also noticed in our study, supporting the hypothesis raised by Natarajan and colleagues that colonization with non-toxigenic strains might be protective against toxigenic isolates (Natarajan et al., 2013), which might also account for the lack of "hypervirulent" RTs in our strain collection.

A remarkably large diversity in RTs was noticed within the isolate set obtained from chicken, with numerous RTs being not reported in any of the poultry studies. However, about 10% of the chicken-derived isolates belonged to RT001 or RT014; two RTs that are frequently encountered in CDI. Both RTs were also present in other poultry studies conducted throughout the world (Supplementary Table 3), indicating that these two RTs are particularly suited to persist in poultry and humans.

The soil-derived *C. difficile* isolate also displayed a high RT diversity, which showed concordance with the RT spectrum observed in the chicken-derived isolate set. Additionally, between chicken- and soil-derived *C. difficile* samples, a relevant clustering in RTs was found. This might be attributed to the fact that the soil samples were taken predominantly on the same locations as the chicken samples (e.g. market places), and that soil contamination might have occurred through chicken faeces.

The most interesting clustering, however, was observed for RT025 and RT084 isolates, respectively. In both RTs, clustering between human and chicken/soil samples could be detected, thus indicating a possible epidemiological link, which might emphasise a zoonotic transmission potential. This is especially astonishing because the samples of the chicken faeces/soil and the humans were collected with a time difference of three years (in 2005 and 2008, respectively), indicating a large genetic stability within those RTs, a possible ongoing transmission, or a common source. According to a public health survey conducted among 470 individuals in Zimbabwe, 299 (58%) reported to keep chicken (CDDEP, 2017). Thus, poultry-associated *C. difficile* transmission is likely to play a more prominent role in this setting.

No resistance towards antibiotics used for treatment of CDI (metronidazole and vancomycin) was found within the samples. Resistance against fluoroquinolones (moxifloxacin), however, was frequently encountered (34% among all isolates). For Zimbabwe, no official governmental monitoring of antimicrobial use is established (CDDEP, 2017). However, other sources report that β-lactam antibiotics (ceftriaxone, penicillin, and amoxicillin, respectively) and ciprofloxacin are in primary use for inpatients, while in animal treatment, tetracyclin and penicillin are frequently employed (CDDEP, 2017). The aforementioned ciprofloxacin use might explain the comparably high rate of moxifloxacin resistance, which is on the same level as the moxifloxacin resistance levels reported for *C. difficile* in Europe and the US with 37% and 38%, respectively (Freeman et al., 2018; Tenover et al., 2012). The largest *C. difficile* AST investigation conducted thus far in Africa (with a representative amount of isolates, i.e. >50) was carried out in a South African tuberculosis hospital and revealed no resistance towards vancomycin, while intermediate resistance (defined as MIC $\geq$2–32 mg/L) towards metronidazole was encountered in 4% of isolates (Kullin et al., 2018). However, resistance towards moxifloxacin and macrolides was highly prevalent with 94% and 75% of isolates, respectively (Kullin et al., 2018). Rifampicin resistance was also very high in the respective study, and reached 99% (Kullin et al., 2018). However, the resistance patterns determined in the cited study are most likely highly influenced by the fact that the investigated patients were treated for tuberculosis and some of the substances with rather high resistance rates (i.e. moxifloxacin, rifampicin, and macrolides) are frequently used for antimycobacterial therapy.

Our study shows some limitations. Only minimal clinical data on humans was available and there was no information about gastrointestinal co-infections and previous antibiotic treatment. The number of isolates is limited, which is however, a common denominator of most *C. difficile* studies conducted across Africa. Furthermore, there is a relevant time gap between the collection of the samples sets of three years (2005 and 2008, respectively). Additionally, not all isolates underwent cgMLST and therefore no comprehensive genotypic resistance testing could be performed.

In conclusion, this study provides a number of new insights into the epidemiology of *C. difficile*: (i) a large strain diversity was evident in samples of humans, chicken and soil; (ii) the majority of RTs found in this Zimbabwean sample set were so far only rarely found in studies from other continents; (iii) a comparably high rate of non-toxigenic strains was noticed, which is in line with studies from other parts of Africa (except for South Africa); (iv) classical "hypervirulent" strains such as RT027 and RT078 were completely absent in this strain set. However, in two strains, binary toxin encoding genes were detected; (v) except for moxifloxacin, antimicrobial resistances were low; and (vi) the observed genetic relatedness between some of the chicken, soil and human *C. difficile* isolates support the proposed potential for zoonotic transmission of this species.

Authors' contribution

Study design: CS, SLB, BG and FKB. Microbiological diagnostics: FB, CS and AM. Wrote the manuscript: FB, CS, BG, MB, SLB and LvM.

All authors have read and approved the final version of the manuscript.

Ethical approval

This initial study was approved by the Ethics Committee of the College of Health Sciences, University of Zimbabwe, Zimbabwe. In this study, only the microbiological characterization of bacterial *C. difficile* isolates was performed and therefore no additional ethical approval was necessary.

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Conflict of interest

None of the authors has a conflict to disclose relevant to this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.plantsci.2004.08.011.

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