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Resistance of *Clostridioides difficile* spores from the NAP_{CR1} strain (Clostridiales: Peptostreptococcaceae) to sodium dichloroisocyanurate

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ABSTRACT

Introduction: *Clostridioides difficile* is a significant cause of diarrhea in hospitals and the community. This bacterial pathogen is transmitted through the ingestion of endospores, which are challenging to eliminate due to intrinsic resistance to a variety of chemical disinfection agents. The well-characterized laboratory strain CD630 displays low virulence, has not caused outbreaks, and is highly susceptible to disinfectants. Nonetheless, a closely related strain termed NAP_{CR1} caused outbreaks in Costa Rica and later became endemic in many hospitals from this country. This strain causes disease through unusual mechanisms and is genotypically distinct from CD630. Consequently, its epidemic potential could be influenced by as yet unknown spore phenotypes, such as increased resistance to disinfectants.

Objective: To determine whether the NAP_{CR1} strain is more resistant to a conventional and highly effective *C. difficile* sporicidal agent than strain CD630 and to identify potential explanatory mechanisms at the genomic level.

Methods: We used an *in vitro* dilution-neutralization method to calculate the sporicidal activity of sodium dichloroisocyanurate (DCC) against purified spores from three subtypes of NAP_{CR1} isolates (LIBA-2945, LIBA-5761, and LIBA-6276), CD630, and a representative of the highly virulent and epidemic NAP1 strain (LIBA-5758). This phenotypic characterization was complemented with a genomics-steered search of polymorphisms in 15 spore- or sporulation-related genes.

Results: Whereas DCC at a final concentration of 0.1 % (w/v) eradicated CD630 endospores with high efficacy (log_{10} reduction factor (LFR) ≥ 5), it only partially inactivated NAP_{CR1} (average LFR range: = 1.77-3.37) and NAP1 endospores (average LRF = 3.58). As hypothesized, the three NAP_{CR1} subtypes tested were more resistant to DCC than strain CD630 (ANOVA, $P < 0.05$), with LIBA-5761 showing the highest level of DCC resistance overall (ANOVA, $P < 0.05$). All three NAP_{CR1} isolates showed large deletions in *bclA1*. Besides, isolates LIBA-5761 and LIBA-6276 had deletions in *bclA2*.

Conclusions: Our *in vitro* tests revealed a differential resistance of spores from the *C. difficile* NAP_{CR1} strain to DCC. They highlight the importance of continuously evaluating the efficacy of deployed disinfection agents against circulating strains and hint to a potential role of structural proteins from the exosporium in resistance to disinfectants in *C. difficile*.

Key words: bacterial endospores; disinfection; sporicidal agent; exosporium proteins.

Clostridioides difficile is a Gram-positive, spore-forming, anaerobic bacterium that may cause diarrhea in susceptible individuals. It colonizes and proliferates in the gastrointestinal tract of humans in response to structural and functional imbalances in the residing microbiota elicited by antibiotic therapy (Martin et al., 2016). Its main virulence factors are toxins TcdB and TcdA, from the family of large clostridial toxins (Khan & Elzouki, 2014). Additional known virulence factors in this bacterium are cysteine proteases, S-layer proteins, and a binary toxin with ADP-ribosyltransferase activity (Kouhsari et al., 2018).

C. difficile infection (CDI) has increased in terms of severity and incidence in the last two decades (Khan & Elzouki, 2014) and become one of the most critical healthcare-associated diseases in developed and developing countries (Khan & Elzouki, 2014).

C. difficile is a highly heterogeneous species, and so, different strains have emerged and predominated in different places of the world. Of all contemporary strains, the NAP/RT27/ST01 strain (MLST Clade 2) has received the most attention because it includes epidemic and hypervirulent isolates whose infections are more severe and linked to higher mortality rates (O'Connor et al., 2009). Furthermore, it is highly resistant to disinfectants due to the activity of efflux pumps and ABC transporters (Dawson et al., 2011). This genotype has circulated in Costa Rica for more than a decade (Guerrero-Araya et al., 2020). It occasionally predominates, yet it has been outnumbered by other lineages (López-Ureña et al., 2016), such as the NAP9 (MLST Clade 4) and the NAP_{CR1} (MLST Clade 1) strains.

The NAP_{CR1}/RT012/ST54 strain was detected for the first time during a *C. difficile* outbreak (Quesada-Gómez et al., 2010) and became endemic in Costa Rican hospitals (López-Ureña et al., 2016). Despite its very small genomic distance to the non-epidemic reference strain CD630 within the *C. difficile* MLST Clade 1, the NAP_{CR1} strain shows increased virulence and causes disease by atypical mechanisms (Quesada-Gómez et al., 2015). Subsequent genomic studies have subdivided it into at least three clusters (I, II, and III) owing to a differential representation of mobile genetic elements in its pangenome (Murillo et al., 2018).

C. difficile infection (CDI) is transmitted through the ingestion of endospores, which are characterized by a distinct multilayer ultrastructure and intrinsic resistance to harsh environmental conditions (Paredes-Sabja et al., 2014). The coat layer of endospores is principally responsible for this trait, as it contains highly impermeable disulfide and dityrosine cross-links and detoxifying enzymes, including superoxide dismutase and peroxiredoxin chitinase (CotE). Moreover, the lipids found in the inner membrane of the endospore are significantly compressed by the spore cortex and consequently show low permeability (Leggett et al., 2012; Gil et al., 2017).

CDI patients excrete endospores that can remain viable in the environment for weeks or months until they are ingested by susceptible hosts (Martin et al., 2016). These developmental forms can contaminate bathrooms, wards, beds, and cleaning and medical equipment at the hospital level. Besides, healthcare staff may act as vectors due to poor hygiene practices, such as inadequate handwash or lack thereof (Durovic et al., 2018).

Household bleach (5.25 % sodium hypochlorite) or chlorine-releasing agents, such as sodium dichloroisocyanurate (DCC) (1 000-5 000 ppm), have proven to be more effective than neutral detergents and quaternary ammonium compounds for environmental decontamination of rooms of patients with CDI and high-touch surfaces (Dubberke et al., 2014; Loo, 2015; Turner & Anderson, 2020). When dissolved in water, DCC reaches a pH between 6-7 and increases the availability of free chlorine in the form of hypochlorous acid (Gallandat et al., 2019). This latter compound, as other oxidizing agents, damages the inner membrane of endospores and blocks their germination (Cortezzo et al., 2004).

Given the importance of spores in CDI spreading (Rineh et al., 2014), we hypothesized that the epidemic potential of the NAP_{CR1} is explained, at least in part, by a greater resistance of its endospores to disinfectants, which has the potential to play important roles in its transmission dynamics. This study was performed to assess this notion and to pinpoint potential explanatory mechanisms based on the availability of whole-genome sequences (WGS) for various NAP_{CR1} isolates.

MATERIALS AND METHODS

Bacterial strains: This study was done with isolates LIBA-2945 (Cluster I), LIBA-5761 (Cluster II), and LIBA-6276 (Cluster III), which represent each of the three clusters into which the NAP_{CR1} strain can be classified (Murillo et al., 2018). Strain CD630 and a NAP1 isolate (LIBA-5758) were also studied as a proxy for *C. difficile* strains with low (Dawson et al., 2011) and high (Ghose, 2013) resistance to disinfectants, respectively.

Preparation of endospores: Bacterial strains were grown at 37 ºC under anaerobic conditions on trypticase soy agar plates supplemented with 0.5 % (w/v) yeast extract. After 5 days of incubation, plates were scratched with sterile loops, and the biomass recovered was suspended in sterile 1X phosphate buffer solution (PBS) containing 1 % bovine serum albumin (BSA, Sigma). These suspensions were homogenized through vigorous vortexing and loaded onto an equal volume of a 100 % (w/v) solution of the non-ionic density gradient medium Histodenz™ (Sigma-Aldrich) to reach a final concentration of 50 % (w/v). Endospores were separated from vegetative cells through centrifugation (10 000 rcf x 10 min). Pellets containing the structures of interest were washed thrice with distilled water to remove Histodenz™ remnants (5 000 rcf x 5 min), and the resulting materials were resuspended in 1X PBS supplemented with 1 % BSA to avoid agglutination (Pizarro-Guajardo et al., 2016). The purity and abundance of viable endospores in these preparations were checked by light microscopy and a plate count method. Brain heart infusion agar plates supplemented with 0.01 % (w/v) sodium taurocholate to induce germination were used in this verification step (Roberts & Mullany, 2016). Endospore suspensions were standardized to contain 10^7 endospores/mL and stored at 4 ºC until use.

Sporicidal assays: The sporicidal activity of 0.1 % (w/v) DCC was determined using a dilution-neutralization method (Vohra & Poxton, 2011). Briefly, a work solution containing 1 000 ppm of DCC prepared from a stock of 10 000 ppm. Later, 100 μL of spores were added to 800 μL of DCC and 100 μL of 1 % BSA to simulate the presence of organic matter in the clinical environment. After 5 min of exposure at room temperature, the activity of DCC was stopped by mixing 100 μL of the mixture with 100 μL of sterile distilled water and 800 μL of 0.5 % sodium thiosulfate as a neutralization agent. Three serial, decimal dilutions of the neutralized mixture were made using 0.85 % sterile physiological saline solution and thereafter 100 μL of each dilution were spread onto Brucella agar plates supplemented with 0.01 % (w/v) sodium taurocholate. After incubation under anaerobiosis at 37 ºC for 72 h, colony counts were recorded and exploited to calculate $\log_{10}$ reduction factors (LRF) (Fraise et al., 2015). A disinfectant agent for clinical use should reach an LRF equal to or greater than 5 to be considered effective (Fraise et al., 2015), which corresponds to a 99.999 % reduction of the initial microbial load. All experiments were performed in triplicate. Differences between disinfectants and strains were examined using one-way ANOVA tests followed by Tukey and Bonferroni post-hoc tests.

Bioinformatic analysis of sporulation genes: Bowtie 2 was used to map trimmed Illumina reads obtained for the LIBA isolates (PRJEB5034, European Nucleotide Archive) to a high-quality, close genome sequence of CD630 (GCF_000009205.2). The resulting bam archives were examined for structural variations in the sequence of genes encoding crucial spore- or sporulation-related proteins

(Table 1). Protein sequences were aligned using MUSCLE and the resulting alignments were visualized with Geneious R10.

TABLE 1
Genes for spore- or sporulation-related proteins scrutinized for structural variants

Gene (locus)	(Possible) Localization
spoIVA (CD2629)	Basement layer [a]
sipL (CD3567)	Basement layer [a]
cotA (CD1613)	Exosporium
cotB (CD1511)	Exosporium
cotCB (CD0598)	Coat and exosporium [a]
cotD (CD2401)	Exosporium
cotE (CD1433)	Exosporium
cotF (CD0597)	Exosporium
cotJB2 (CD2400)	ND[b]
cotG (CD1567)	Exosporium
sodA (CD1631)	Coat
bclA1 (CD0332)	Exosporium
bclA2 (CD3230)	Exosporium
bclA3 (CD3349)	Exosporium
cdeC (CD1067)	Exosporium

[a]: as yet unconfirmed; [b]ND: not determined.

RESULTS

Sporicidal effect of disinfectants: As indicated in Fig. 1, DCC fully inactivated spores from strain CD630 (LRF ≥ 5) and induced undistinguishable reductions of intermediate magnitude for the NAP_{CR1} isolates LIBA-2945 (LRF = 3.64 ± 0.63) and LIBA-6276 (LRF = 3.37 ± 0.46), and the NAP1 isolate LIBA-5758 (LRF = 3.58 ± 0.40). With an LRF that differed by almost two orders of magnitude (1.77 ± 0.47), LIBA-5761 exhibited the highest level of *in vitro* resistance to DCC overall (Fig. 1). The NAP_{CR1} isolates were invariably more resistant to DCC than strain CD630 (ANOVA, $P < 0.05$).

Bioinformatic analyses: Compared to CD630, all three NAP_{CR1} isolates exhibited a large deletion in the aminoacid sequence of BclA1. Besides, LIBA-5761 (Cluster II) and LIBA-6276 (Cluster III), showed a deletion of three aminoacid residues in BclA2 (Fig. 2, Fig. 3) No differences were observed in the other analyzed genes.

DISCUSSION

This is the first investigation that addresses the response to disinfectants of endospores from the *C. difficile* NAP_{CR1} strain. In agreement with our working hypothesis, and possibly in line with its epidemic potential, the NAP_{CR1} strain was found to be more resistant to DCC than strain CD630. This conclusion was reached by an *in vitro* experiment; hence it could be strengthened by performing similar tests on surfaces contaminated with different

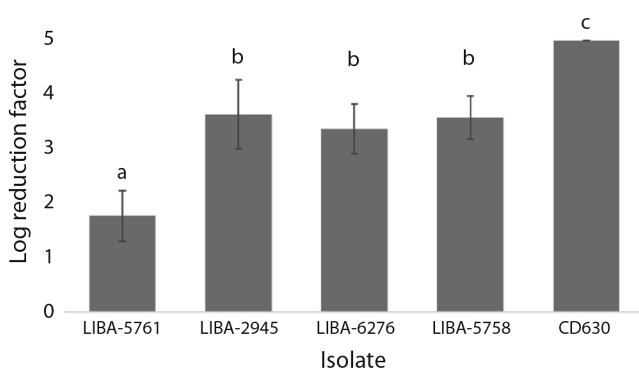

Fig. 1. Activity of DCC against *C. difficile* endospores. Endospore preparations from three subtypes of the NAP_{CR1} strain (LIBA-5761, LIBA-2945, LIBA-6276), a NAP1 strain (LIBA-5758), and a reference laboratory strain (CD630), were tested by triplicate using a dilution-neutralization method. Results were expressed as average log_{10} reduction factors (LRF), whereby an LRF = 5 indicates a 99.999 % reduction in the original number of endospores. A disinfectant should reach this threshold value to be considered effective. Error bars represent standard deviations. Different letters above the bars indicate a statistically significant difference at $P < 0.05$ (one-way ANOVA).

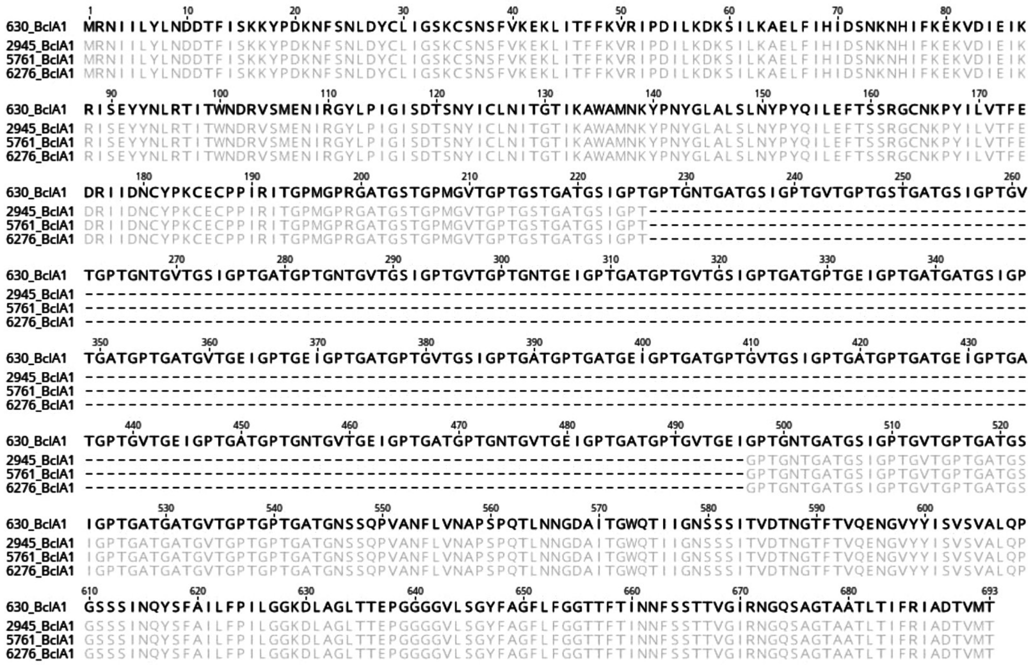

Fig. 2. Alignment of the predicted BclA1 protein sequences of strain 630 (reference, top) and three subtypes of the NAP_{CR1} strain. Agreements and deletions to the reference sequence appear masked or as hyphens, respectively. The numbering of the sequence alignment is based on the sequence of strain 630.

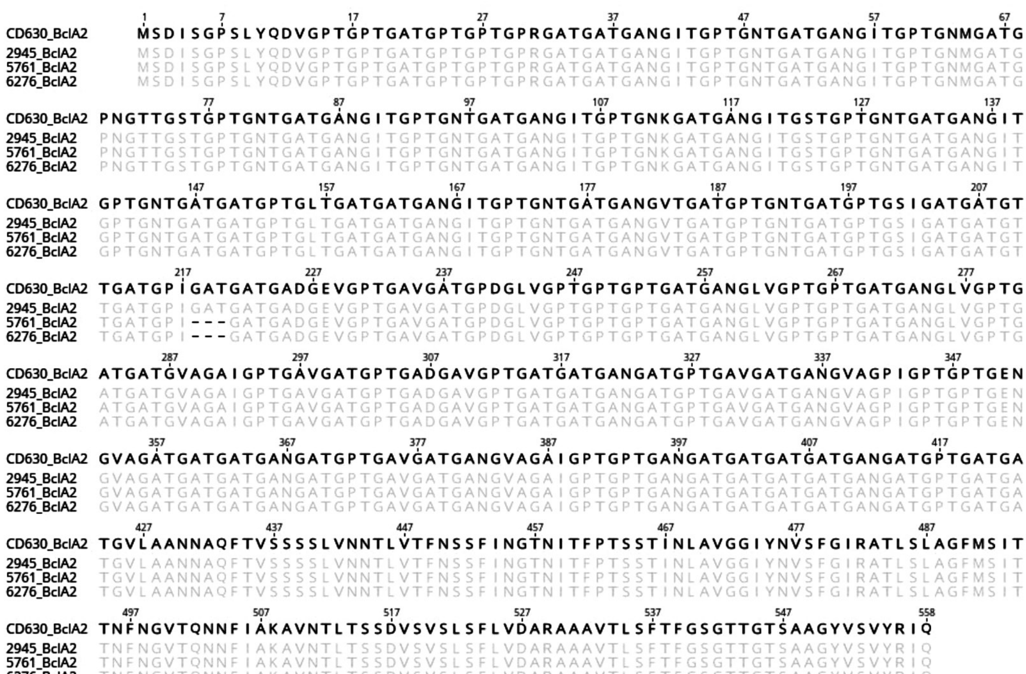

Fig. 3. Alignment of the predicted BclA2 protein sequences of strain 630 (reference, top) and three subtypes of the NAP_{CR1} strain. Agreements and deletions to the reference sequence appear masked or as hyphens, respectively. The numbering of the sequence alignment is based on the sequence of strain 630.

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loads and types of organic matter. In most cases, DCC did not reach the LRF ≥ 5 threshold required for a disinfectant agent to be considered effective under our experimental conditions (5 min exposure time, 1 000 ppm). This apparent caveat can be solved by the application of DCC in higher concentrations and for longer contact times (Barbut, 2015). In any case, it is advisable to stimulate the development of alternative compounds on account of the toxicity and corrosiveness of DCC and other chlorine-release agents (Ungurs et al., 2011; Balsells et al., 2016).

This study shows that no disinfection protocol works equally well for all *C. difficile* strains and even for isolates of the same strain. This finding emphasizes the importance of evaluating multiple field-isolates together with reference strains. Moreover, it justifies continuous typing and testing of the circulating isolates at a given time and place, as they may have been exposed to undetermined selective pressures.

The susceptibility of one NAP_{CR1} subtype to DCC was unexpectedly low (LIBA-5761, Cluster III). However, this trait could not be linked to a distinct profile of sequence variants in spore- and sporulation-related genes. Of all genes studied, only those for the collagen-like exosporium proteins BclA1 and BclA2 showed sequence polymorphisms. *C. difficile* has three *bclA* genes (*bclA1*, *bclA2,* and *bclA3*), and their collagen-like regions are somewhat similar to those found in other sporulated bacteria such as *Bacillus cereus* and *B. anthracis* (Pizarro-Guajardo et al., 2014). BclA1 has been characterized to some extent, but even less is known about BclA2 and BclA3.

BclA1 forms stable high molecular mass complexes with other exosporium proteins (Pizarro-Guajardo et al., 2014) and increases the hydrophobicity of the exosporium in *B. anthracis* (Phetcharaburanin et al., 2014). Based on this knowledge, we predict that the observed deletions in *bclA1* and *bclA2* lead to the formation of a more robust exosporium layer through increased cross-linking and water repellency, hampering the accessibility of DCC to its internal target in endospores (Cortezzo et al., 2004). This notion could be confirmed through a comprehensive comparison of the composition of the exosporium of strains CD630 and NAP_{CR1} by electron microscopy and proteomics. On the other hand, since isolates with identical gene deletion profiles displayed different levels of DCC susceptibility, it is plausible that the phenotype of LIBA-5761 is multifactorial and influenced by physiological factors and the activity of efflux pumps or ABC transporters.

Our results highlight the importance of continuously evaluating the efficacy of deployed disinfection agents against circulating strains and hint to a potential role of exosporium proteins in resistance to disinfectants in *C. difficile*. The relevance of these findings is twofold. On the one hand, it may guide decision-makers and contribute to resource optimization in hospitals. On the other, it deepens our understanding of the functional diversity of *C. difficile* and its role in disease control.

Ethical statement: The authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that they followed all pertinent ethical and legal procedures and requirements. All financial sources are fully and clearly stated in the acknowledgements section. A signed document has been filed in the journal archives.

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RESUMEN

Resistencia de esporas de *Clostridioides difficile* de la cepa NAP_{CR1} (Clostridiales: Peptostreptococcaceae) al dicloroisocianurato de sodio

Introducción: *Clostridioides difficile* es una causa importante de diarrea a nivel hospitalario y comunitario. Esta bacteria se transmite por medio de la ingestión de endosporas, las cuales son difíciles de erradicar por su resistencia intrínseca a diferentes agentes químicos de desinfección. La cepa de referencia CD630 está bien caracterizada, es poco virulenta, no ha causado brotes, y es altamente susceptible a los desinfectantes. Además, pertenece al mismo clado MLST y es filogenéticamente muy cercana a la cepa NAP_{CR1}. Sin embargo, solo la última ha causado brotes en Costa Rica y se ha convertido en una cepa endémica en varios hospitales locales. La cepa NAP_{CR1} causa enfermedad por mecanismos poco usuales y es genotípicamente diferente a la cepa CD630. Por lo tanto, su potencial epidémico podría estar influenciado por cambios fenotípicos en sus esporas, como una resistencia incrementada a los desinfectantes.

Objetivo: Determinar si la cepa NAP_{CR1} presenta mayor resistencia que CD630 a un desinfectante de alta eficacia utilizado a nivel hospitalario y dilucidar posibles mecanismos a nivel genómico.

Métodos: Se utilizó el método de dilución-neutralización para evaluar la actividad esporicida *in vitro* del dicloroisocianurato de sodio (DCC) contra esporas de 3 subtipos de la cepa NAP_{CR1} (LIBA-2945, LIBA-5761, y LIBA-6276), CD630 y un aislamiento representativo de la cepa epidémica e hipervirulenta NAP1 (LIBA-5758). Esta caracterización fenotípica fue complementada con una búsqueda genómica de polimorfismos en 15 genes relacionados con la estructura de la endospora o el proceso de esporulación.

Resultados: El DCC a una concentración final de 0.1 % (p/v) erradicó las endosporas de la cepa CD630 con gran eficacia (factor de reducción logarítmica; FRL ≥ 5) y eliminó parcialmente las de las cepas NAP_{CR1} (FRL promedio = 1.77-3.64) y NAP1 (FRL promedio = 3.58). El perfil de susceptibilidad del aislamiento NAP_{CR1} LIBA-5761 fue único, ya que mostró un mayor nivel de resistencia hacia el DCC que los otros aislamientos NAP_{CR1} y la cepa NAP1 examinada (ANOVA, $P < 0.05$). Los tres aislamientos NAP_{CR1} mostraron deleciones en *bclA1* y los aislamientos LIBA-5761 y LIBA-6276 tenían deleciones adicionales en *bclA2*.

Conclusiones: Nuestros experimentos *in vitro* confirman la resistencia incrementada a los desinfectantes de la cepa NAP_{CR1} y una susceptibilidad diferencial en sus tres subtipos. Adicionalmente, señalan la importancia de evaluar continuamente la eficacia de los desinfectantes contra cepas circulantes y asignan un posible papel en la resistencia a los desinfectantes gracias a las proteínas del exosporio de *C. difficile*.

Palabras clave: endosporas bacterianas; desinfección; agentes esporicidas; proteínas de exosporio.

REFERENCES

Balsells, E., Filipescu, T., Kyaw, M.H., Wiuff, C., Campbell, H., & Nair, H. (2016). Infection prevention and control of *Clostridium difficile*: a global review of guidelines, strategies and recommendations. *Journal of Global Health, 6*(2), 020410. https://doi.org/10.7189/jogh.06.020410

Barbut, F. (2015). How to eradicate *Clostridium difficile* from the environment. *Journal of Hospital Infection, 89*, 287–295. https://doi.org/10.1016/j.jhin.2014.12.007

Cortezzo, D.E., Koziol-Dube, K., Setlow, B., & Setlow, P. (2004). Treatment with oxidizing agents damages the inner membrane of spores of *Bacillus subtilis* and sensitizes spores to subsequent stress. *Journal of Applied Microbiology, 97*, 838–852. https://doi.org/10.1111/j.1365-2672.2004.02370.x

Dawson, L.F., Valiente, E., Donahue, E.D., Birchenough, G., & Wren, B.W. (2011). Hypervirulent *Clostridium difficile* PCR-Ribotypes exhibit resistance to widely used disinfectants. *PLoS ONE*, *6*(10), e25754. https://doi.org/10.1371/journal.pone.0025754

Dubberke, E.R., Carling, P., Carrico, R., Donskey, C.J., Loo, V.G., McDonald, L.C., Maragakis, L.L., Sandora, T.J., Weber, D.J., Yokoe, D.S., & Gerding, D.N. (2014). Strategies to prevent *Clostridium difficile* infections in acute care hospitals: 2014 update. *Infection Control & Hospital Epidemiology, 35*(6), 628–645. https://doi.org/10.1086/676023

Durovic, A., Widmer, A.F., & Tschudin-Sutter, S. (2018). New insights into transmission of *Clostridium difficile* infection - a narrative review. *Clinical Microbiology and Infection, 24*, 483–492. https://doi.org/10.1016/j.cmi.2018.01.027

Fraise, A.P., Wilkinson, M.A.C., Bradley, C.R., Paton, S., Walker, J., Maillard, J., Wesgate, R.L., Hoffman, P., Coia, J., Woodall, C., Fry, C., & Wilcox, M. (2015). Development of a sporicidal test method for *Clostridium difficile*. *Journal of Hospital Infection*, *89*, 2–15. https://doi.org/10.1016/j.jhin.2014.09.014

Gallandat, K., Stack, D., String, G., & Lantagne, D. (2019). Residual maintenance using sodium hypochlorite, sodium dichloroisocyanurate and chlorine dioxide in laboratory waters of varying turbidity. *Water, 11*(1309), 1–14. https://doi.org/10.3390/w11061309

Ghose, C. (2013). *Clostridium difficile* infection in the twenty-first century. *Emerging Microbes and Infections, 2,* 1–8. https://doi.org/10.1038/emi.2013.62

Gil, F., Lagos-Moraga, S., Calderón-Romero, P., Pizarro-Guajardo, M., & Paredes-Sabja, D. (2017). Updates on *Clostridium difficile* spore biology. *Anaerobe*, *45*, 3–9. https://doi.org/10.1016/j.anaerobe.2017.02.018

Guerrero-Araya, E., Meneses, C., Castro-Nallar, E., Guzmán, A.M., Álvarez-Lobos, M., Quesada-Gómez, C., Paredes-Sabja, D., & Rodríguez, C. (2020). Origin, genomic diversity and microevolution of the *Clostridium difficile* B1/NAP1/RT027/ST01 strain in Costa Rica, Chile, Honduras and Mexico. *Microbial Genomics, 6*(5), e000355. https://doi.org/10.1099/mgen.0.000355

Khan, F.Y., & Elzouki, A.N. (2014). *Clostridium difficile* infection: a review of the literature. *Asian Pacific Journal of Tropical Medicine, 7*(1), S6–S13. https://doi.org/10.1016/S1995-7645(14)60197-8

Kouhsari, E., Abbasian, S., Sedighi, M., Yaseri, H.F., Nazari, S., Bialvaei, A.Z., Dahim, P., Mirzaei, E.Z., & Rahbar, M. (2018). *Clostridium difficile* infection: a review. *Reviews in Medical Microbiology, 29*, 103–109. https://doi.org/10.1097/MRM.0000000000000135

Leggett, M.J., McDonnell, G., Denyer, S.P., Setlow, P., & Maillard, J.Y. (2012). Bacterial spore structures and their protective role in biocide resistance. *Journal of Applied Microbiology, 113*, 485–498. https://doi.org/10.1111/j.1365-2672.2012.05336.x

Loo, V.G. (2015). Environmental interventions to control *Clostridium difficile*. *Infectious Disease Clinics, 29*, 83–91. https://doi.org/10.1016/j.idc.2014.11.006

López-Ureña, D., Quesada-Gómez, C., Montoya-Ramírez, M., Gamboa-Coronado, M.D.M., Somogyi, T., Rodríguez, C., & Rodríguez-Cavallini, E. (2016). Predominance and high antibiotic resistance of the emerging *Clostridium difficile* genotypes NAP_{CR1} and NAP9 in a Costa Rican hospital over a 2-year period without outbreaks. *Emerging Microbes and Infections, 5*(1), 1–5. https://doi.org/10.1038/emi.2016.38

Martin, J., Monaghan, T.M., & Wilcox, M.H. (2016). *Clostridium difficile* infection: epidemiology, diagnosis and understanding transmission. *Nature Reviews: Gastroenterology & Hepatology*, *13*, 206–216. https://doi.org/10.1038/nrgastro.2016.25

Murillo, T., Ramírez-Vargas, G., Riedel, T., Overmann, J., Andersen, J.M., Guzmán-Verri, C., Chaves-Olarte, E., & Rodríguez, C. (2018). Two groups of cocirculating, epidemic *Clostridiodes difficile* strains microdiversify through different mechanisms. *Genome Biology and Evolution, 10*(3), 982–998. https://doi.org/10.1093/gbe/evy059

O'Connor, J.R., Johnson, S., & Gerding, D.N. (2009). *Clostridium difficile* infection caused by the epidemic B1/NAP1/027 strain. *Gastroenterology, 136*, 1913–1924. https://doi.org/10.1053/j.gastro.2009.02.073

Paredes-Sabja, D., Shen, A., & Sorg, J.A. (2014). *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. *Trends in Microbiology, 22*(7), 406–416. https://doi.org/10.1016/j.tim.2014.04.003

Phetcharaburanin, J., Hong, H.A., Colenutt, C., Bianconi, I., Sempere, L., Permpoonpattana, P., Smith, K., Dembek, M., Tan, S., Brisson, M.C., Brisson, A.R., Fairweather, N.F., & Cutting, S.M. (2014). The spore-associated protein BclA1 affects the susceptibility of animals to colonization and infection by *Clostridium difficile*. *Molecular Microbiology, 92*(5), 1025–1038. https://doi.org/10.1111/mmi.12611

Pizarro-Guajardo, M., Calderón-Romero, P., Castro-Córdoba, P., Mora-Uribe, P., & Paredes-Sabja, D. (2016). Ultrastructural variability of the exosporium layer of *Clostridium difficile* spores. *Applied and Environmental Microbiology*, *82*(7), 2202–2209. https://doi.org/10.1128/AEM.03410-15

Pizarro-Guajardo, M., Olguín-Araneda, V., Barra-Carrasco, J., Brito-Silva, C., Sarker, M., & Paredes-Sabja, D. (2014). Characterization of the collagen-like exosporium protein, BclA1, of *Clostridium difficile* spores. *Anaerobe*, *25*, 18–30. https://doi.org/1016/j.anaerobe.2013.11.003

Quesada-Gómez, C., Gamboa-Coronado, M.D.M., Rodríguez-Cavallini, E., Du, T., Mulvey, M.R., Villalobos-Zúñiga, M., Boza-Cordero, R., & Rodríguez, C. (2010). Emergence of *Clostridium difficile* NAP1 in Latin America. *Journal of Clinical Microbiology, 48*(2), 669–670. https://doi.org/10.1128/JCM.02196-09.

Quesada-Gómez, C., López-Ureña, D., Acuña-Amador, L., Villalobos-Zuñiga, M., Du, T., Freire, R., Guzmán-Verri, C., Gamboa-Coronado, M.D.M., Lawley, T.D., Moreno, E., Mulvey, M.R., Brito, G.A., Rodríguez-Cavallini, E., Rodríguez, C., & Chaves-Olarte, E. (2015). Emergence of an outbreak-associated *Clostridium difficile* variant with increased virulence. *Journal of Clinical Microbiology, 53*(4), 1216–1226. https://doi.org/10.1128/JCM.03058-14

Rineh, A., Kelso, M.J., Vatansever, F., Tegos, G.P., & Hamblin, M.R. (2014). *Clostridium difficile* infection: molecular pathogenesis and novel therapeutics. *Expert Review of Anti-infective Therapy, 12*(1), 131–150. https://doi.org/10.1586/14787210.2014.866515

Roberts, A.P., & Mullany, P. (2016). *Clostridium difficile Methods and Protocols*. Humana Press.

Turner, N.A., & Anderson, D.J. (2020). Hospital infection control: *Clostridoides difficile*. *Clinics in Colon and Rectal Surgery, 33*, 98–108. https://doi.org/10.1055/s-0040-1701234

Ungurs, M., Wand, M., Vassey, M., O-Brien, S., Dixon, D., Walker, J., & Sutton, J.M. (2011). The effectiveness of sodium dichloroisocyanurate treatments against *Clostridium difficile* spores contaminating stainless steel. *American Journal of Infection Control, 39*(3), 199–205. https://doi.org/10.1016/j.ajic.2010.07.015

Vohra, P., & Poxton, I.R. (2011). Efficacy of decontaminants and disinfectants against *Clostridium difficile*. *Journal of Medical Microbiology*, *60*, 1218–1224. https://doi.org/10.1099/jmm.0.030288-0