Title: Molecular and biochemical characterization of human pathogenic *Clostridioides difficile* strains isolated from hospitalized patients in the Silesia region.

Introduction: *Clostridioides difficile* is a spore-forming, anaerobic bacillus and the leading cause of healthcare-associated infections in Poland. Although data from the State Sanitary Inspection (PIS) show a decrease in this pathogen's involvement in epidemic outbreaks (from over 50% in 2021 to 27% in 2024), it remains the most frequent source of hospital epidemics. A serious threat is the emergence of a new, toxin-producing ribotype – RT955.

The aim of this study was the molecular and biochemical characterization of *C. difficile* strains.

Material and Methods: From 208 stool samples from patients with antibiotic-associated diarrhea (previously positive for GDH), 191 *C. difficile* strains were cultured. The procedure included inoculation on selective media CDIFF and CLO, followed by biochemical identification Multiplex PCR (mPCR) was used to detect genes encoding GDH, toxins A and B, and binary toxin in the *C. difficile* isolates. Each strain was ribotyped, and its antimicrobial resistance profile was assessed. Collected clinical data of the patients were analyzed. In two strains – 500/12 and CKH08 – sporulation and quantitative concentrations of toxins A and B were evaluated using the ELISA technique.

Results: The presence of toxins A and B was confirmed in 185/191 (96.9%) of *C. difficile* strains, while binary toxin was detected in 137/191 (71.7%). RT027 accounted for 107/191 (56.0%) of the strains, RT955 for 16/191 (8.4%), and RT014 for 12/191 (6.3%). Six strains remained unclassified. All isolated *C. difficile* strains (n=191) were susceptible to vancomycin. Resistance to metronidazole was found in 17 cases (8.9%). Strains in which the *ermB* gene was detected (50.3%) were resistant to clindamycin. The presence of the phi027 prophage positively influences the initiation of sporulation; however, its removal results in an increase in toxin A production and a decrease in toxin B levels compared to strain 500/12.

Conclusions: The main cause of hospital outbreaks in the Silesian Voivodeship are toxigenic strains of *C. difficile*. The spread of clonal RT955 strains necessitates strict, prospective epidemiological surveillance of this pathogen in Poland and Europe. The reported resistance to metronidazole among the isolated *C. difficile* strains indicates the necessity of introducing routine ribotyping and antimicrobial susceptibility testing into diagnostic standards.

Keywords: *Clostridioides difficile*, toxigenic strains, ribotype, epidemic outbreak, drug resistance