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A novel immunoproteomics method for identifying in vivo-induced *Campylobacter jejuni* antigens using pre-adsorbed sera from infected patients ☆

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ABSTRACT

Background: *Campylobacter jejuni* is an important food-borne and zoonotic pathogen with a worldwide distribution. Humans and chickens are hosts of this pathogen. At present, there is no ideal vaccine for controlling human campylobacteriosis or the carriage of *C. jejuni* by chickens. Bacterial in vivo-induced antigens are useful as potential vaccine candidates and biomarkers of virulence.

Methods: In this study, we developed a novel systematic immunoproteomics approach to identify in vivo-induced antigens among the total cell proteins of *C. jejuni* using pre-adsorbed sera from patients infected with *C. jejuni*.

Results: Overall, 14 immunoreactive spots were probed on a PVDF membrane using pre-adsorbed human sera against *C. jejuni*. Then, we excised these protein spots from a duplicate gel and identified using MALDI–TOF MS. In total, 14 in vivo-induced antigens were identified using PMF and BLAST analysis. The identified proteins include CadF (CadF-1 and CadF-2), CheW, TufB, DnaK, MetK, LpxB, HslU, DmsA, PorA, ProS, CJBH_0976, CSU_0396 and hypothetical protein cje135_05017. Real-time RT-PCR was performed on 9 genes to compare their expression levels in vivo and in vitro. The data showed that 8 of the 9 analyzed genes were significantly upregulated in vivo relative to in vitro.

Conclusion: We successfully developed a novel immunoproteomics method for identifying in vivo-induced *Campylobacter jejuni* antigens by using pre-adsorbed sera from infected patients.

General significance: This new analysis method may prove to be useful for identifying in vivo-induced antigens within any host infected by bacteria and will contribute to the development of new subunit vaccines.

1. Introduction

Campylobacter jejuni is a thermotolerant and microaerophilic spiral Gram-negative bacterium [1] that has emerged as the leading cause of human bacterial food-borne diarrheal disease worldwide, especially in many industrialized countries [2]. Children are especially susceptible to *C. jejuni* in both developed and developing countries [3,4]. *C. jejuni* can colonize the lower intestines of many hosts, including humans, chickens, cattle, sheep [5], dogs, and wild birds [6]. In humans, *C. jejuni* infection can induce self-limiting diarrhea [3]. Guillain–Barré syndrome (GBS) is an acute polyneuropathy [1,7] with a global incidence of 0.6–4 cases per 100,000 people. This disease generally occurs after infection by *C. jejuni* from contaminated food or water [3,8–10]. Evidence suggests that GBS results from molecular mimicry due to the similarity between GM1 and GD1a gangliosides and the lipooligosaccharides (LOS) of *C. jejuni* [2,11]. There are a number of *Campylobacter* antigens during infection, including in vivo or in vitro expressed virulence-associated factors. However, the antibodies response to diarrhea is different to GBS infected by *C. jejuni* [12,13]. Unlike other diarrhea-causing bacteria, *C. jejuni* does not express many classical virulence factors, such as pilus structures [1,4]. However, *C. jejuni* contains O- and N-linked glycosylation systems. Due to the limited information on the pathogenicity of *C. jejuni* within its hosts, an effective vaccine for *C. jejuni*, which may help to control infections, is lacking [14].

Abbreviations: *C. jejuni*, *Campylobacter jejuni*; MALDI–TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; PMF, peptide mass fingerprinting; GBS, Guillain–Barré syndrome; LOS, lipooligosaccharides; NMRC, Naval Medical Research Center; MBP, maltose-binding protein; DOC, sodium deoxycholate; 2-DE, two-dimensional echocardiography; CCDA, Campy blood-free selective medium; IEF, isoelectric focusing; HRP, horseradish peroxidase; IVET, in vivo expression technology; IVIAT, in vivo-induced antigen technology; STM, signature-tagged mutagenesis; DFI, differential fluorescence induction

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The development of an effective *C. jejuni* vaccine has been a research goal for many years, and most vaccine studies have concentrated on whole-cell and subunit vaccines [14]. The Naval Medical Research Center (NMRC) used killed *C. jejuni* cells as vaccine to immunize mouse and monkey models, and their results showed its significant protective effect. Unfortunately, this vaccine is not used in clinical settings even though a Phase II clinical trial was conducted and showed efficacy [14,15]. Subunit vaccines are the safest vaccines, but their efficacy is lower than whole-cell vaccines. The maltose-binding protein (MBP)-FlaA protein vaccine was proven to protect mice from colonization, but its application in immunoprophylaxis was problematic [16]. Antigens that are induced in vivo when pathogens infect their hosts may be crucial factors, and some may elicit a protective effect [14].

In vivo-induced antigens are important virulence-associated factors, and these proteins are considered major pathogenicity-related molecules involved in the interactions between hosts and microorganisms. Due to the lack of an ideal animal model to study the pathogenicity of *C. jejuni* in humans, the identification of in vivo-induced antigens was hampered for a long time. In 2008, Malik-Kale [17] demonstrated that culturing *C. jejuni* with sodium deoxycholate (DOC) induces the expression of virulence genes, and these culture conditions mimic the in vivo environment, obviating the need for animal models.

Immunoproteomics has been used to identify immunoreactive proteins from many microorganisms, such as *Streptococcus suis* [18,19] and *Helicobacter pylori* [20]. However, there is no report of the analysis of *C. jejuni* using immunoproteomics. In this study, to remove the antibodies induced by in vitro antigens, patient sera were pre-adsorbed 6 times with *C. jejuni* whole cells, cell lysates and secreted protein. We used these pre-adsorbed sera to probe two-dimensional electrophoresis (2-DE) gels of *C. jejuni* whole-cell proteins from *C. jejuni* cultured in the presence of DOC to mimic the in vivo environment. These identified proteins were assumed to be in vivo-induced antigens and identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS). A schematic diagram of the detection method for the in vivo-induced antigens is shown in Fig. 1. The identified in vivo-induced factors may be candidate antigens for preventing disease caused by *C. jejuni* or diagnostic markers. The novel method was embodied in 3 fields of improvement. Firstly, it does not depend on *Escherichia coli* expression of proteins in vitro away, avoiding the irregular protein expression. Secondly, DOC culture conditions mimic the in vivo environment, obviating the need for animal models. Thirdly, the method is a systematic immunoproteomics approach to identify in vivo-induced antigens among the total cell proteins with *C. jejuni*.

2. Materials and methods

2.1. Bacteria and culture conditions

C. jejuni strain NCTC 11168, which was kindly provided by Dr. Wanbang Sun from Zunyi Medical College in China, was cultured on Campy blood-free selective medium (CCDA) (Oxoid Ltd., Basingtoke, Hampshire, England) plates at 42 °C under microaerobic conditions. To induce the expression of in vivo-induced antigens, *C. jejuni* strain NCTC 11168 was cultured on CCDA medium containing 0.1% sodium deoxycholate (DOC, Sigma, USA) [17] (referred to as DOC plates) for 15 h under microaerobic conditions.

2.2. Preparation of pre-adsorbed anti-sera

In this study, human sera were obtained from 7 clinical volunteers (Subei Hospital of Yangzhou, China) who had previously suffered from repeated diarrhea due to *C. jejuni* infection. These patients were more than 50 years old, and although they reported suffering from repeated episodes of diarrhea during the interview, they never sought medical advice until general health survey. Human sera experiments were conducted in accordance with national guidelines and were approved by Subei Hospital of Yangzhou and each patient. The antibody levels of these sera were determined using an indirect ELISA kit (SERION ELISA classic CAMPYLOBACTER JEJUNI IgG, Virion\Serion, Wurzburg, Germany). Sera that were pooled in equal portions were sequentially adsorbed using *C. jejuni* whole NCTC 11168 cells, ultrasonic lysates, inactivated lysates and secreted proteins. Firstly, whole cells mixing with sera was performed on a rocking platform (Incubator Shaker, Crystal, China) at 4 °C for 1 h and repeated 6 times. The subsequent adsorption steps were performed 6 times using PVDF membranes coated with the in vitro antigens. Each adsorption step was performed at 4 °C on a rocking platform for 1 h. Aliquots of the final adsorbed sera were stored at −80 °C for 2-D blotting. Indirect ELISAs were performed to evaluate the adsorption of the anti-in vitro antigen antibodies in the sera by coating the plates with *C. jejuni* NCTC 11168 whole cells, lysates, CjaA, recombinant FlaA370 protein (prepared previously in our laboratory) and total flagellum protein [21].

2.3. Protein sample extraction

Whole-cell proteins from *C. jejuni* were extracted according to the protocol of Zhang [22], with minor modifications. Briefly, *C. jejuni* cells

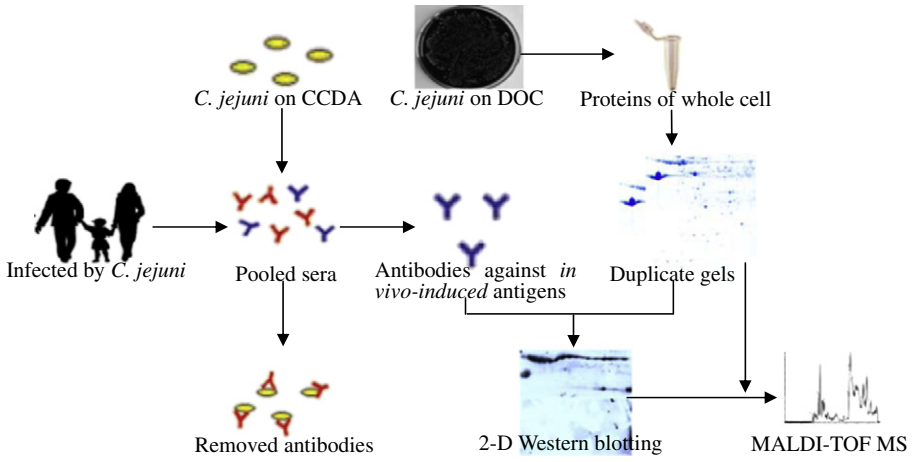

Fig. 1. Schematic diagram of the immunoproteomics assay to identify in vivo-induced antigens. Sera from 7 patients infected with *C. jejuni* were pooled and pre-adsorbed to enrich antibodies that recognize in vivo-induced antigens produced by *C. jejuni* grown on DOC plates, which mimic in vivo conditions. Then, the pre-adsorbed sera were used to probe 2-DE gels of whole proteins from *C. jejuni* cultured under DOC culture conditions. Spots that appeared in the 2-D Western blotting were assumed to be in vivo-induced antigens expressed after *C. jejuni* infects humans. DOC plates were prepared using CCDA medium plus 0.1% sodium deoxycholate.