

CHARACTERIZATION OF PLASMIDS ISOLATED FROM BACTERIAL FLORA OF CRC PATIENTS – PROOF OF CONCEPT

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Abstract

Introduction: Colorectal cancer (CRC) is one of the leading oncogenic disorders, both in terms of incidence and mortality. The etiology of the disease is certainly multifactorial. Various risk factors like alcohol consumption, smoking, CRC family history, inflammatory bowel disease, hormone therapy, aspirin/nonsteroidal anti-inflammatory drugs use, higher body mass index, consumption of red and/or processed meat, insufficient physical activity, and decreased intake of fruit and vegetables have been pointed out; however, there is not enough support evidence for a single particular causative mechanism. Recently, gut bacterial microbiota has been shown to influence significantly the pathogenesis of CRC. However, little attention is paid to the putative impact of plasmids in gut flora.

Material and methods: We have designed and tested the workflow for semi-selective isolation and amplification of random circular sequences. The exploitation of rolling circle amplification (RCA) with a random hexamers protocol is crucial for the outcome. **Results:** Our results suggest that it is possible to isolate and amplify plasmid DNA from gut flora and further process, sequence, and identify them. **Discussion:** Little is known about the interactions between bacterial plasmids and human cells. The collection of plasmid sequencing data and the comparison of CRC patients and healthy control sequences can be the first step to elucidating this phenomenon.

Keywords: CRC, gut bacterial flora, plasmids

INTRODUCTION

Colorectal cancer (CRC), with between one and two million new cases every year, is undoubtedly one of the most common oncological diseases worldwide (1, 2). It is the third most common cancer diagnosed and the fourth most common reason for cancer-associated death (1,3). The global distribution of newly diagnosed cases is unequal, and the incidence rates are highest in developed countries; however, due to the “westernization” of diet and lifestyle in developing countries, it is expected to significantly increase the global burden of disease by 2030 (4). Also, an alarming growing trend in early-onset CRC in young individuals has been observed in recent years. Particularly, the incidence in individuals under 50 has been increasing by 2% per year (5). Nevertheless, this observation can be partially attributed to the rapid development of new approaches to early screening (6).

The pathogenesis of colorectal cancer is multifactorial. Several mutations in genes, such as *APC*, *KRAS*, *BRAF*, *P53*, *PIK3CA*, *SMAD4*, *ARID1A*, *MYC*, and others, have been associated with the development of CRC (7–11). Also, the involvement of lncRNAs like *GLCC1*, *LINRIS*, and many more have been described (12–14).

Both lifestyle and genetic factors play an important role in the etiology of CRC. The influence of nutrition on initiating the pathological process has been studied extensively

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(3,15). Presumed risk factors associated with the development of CRC include alcohol consumption, cigarette smoking, CRC family history, inflammatory bowel disease (IBD), former and current postmenopausal hormone therapy (HT), aspirin/nonsteroidal anti-inflammatory drugs (NSAIDs) usage, higher body mass index (BMI), frequent consumption of red and/or processed meat, insufficient physical activity (PA), and a decreased intake of fruit and vegetables (16, 17). However, the precise causative factors are yet unknown. For example, eating white meat or fish is not associated with a high risk and may even reduce the incidence of CRC. The influence of saturated fat, protein, iron, heterocyclic amines produced by cooking, N-nitroso compounds, and increased levels of bile acids in the colonic lumen has been considered (18). The work of zur Hausen outlined a hypothesis that not directly the consumption of undercooked red meat but the presence and survival of certain – for now elusive – causative agent in undercooked meat may play an important role in pathogenesis (19).

Lately, another factor – gut bacterial flora – has gained more attention. Indeed, it has been shown that the species composition and the metabolic activity of the intestine flora of CRC patients differ significantly from those of healthy controls (20–22). The correlation between the gut microbiome status and the presence of CRC was shown to be sensitive and specific enough to serve as a reliable biomarker for this malignancy (23). On the other hand, gut microbiota can have a protective effect. For example, gut flora can enhance the immune system response via the stimulation of chemokine production by CRC cells, thus increasing the recruitment of beneficial T cells into tumor tissues (24). Also, the species *Clostridium butyricum* was shown to reprogram the proliferation, migration, stemness, and tumor growth in CRC by regulating pivotal signal molecules, including MYC (25).

The recent discovery of viroid-like elements denoted “obelisks” illustrates that not enough attention is paid to the structures that are hosted by bacteria. These entities remained unnoticed despite being widely spread in human colonizing microbiota (26). Needless to say, plasmids are generally known to be present in bacteria, archaea, and even some eucaryotic organisms, and they are classified into several classes by their function; however, not much attention is given to this fact in the context of a possible interaction with cells of the human intestine tract. Few studies analyzed meta-genomic data and suggested particular plasmids to be used as biomarkers (27).

Little is known about the possible interactions between bacterial plasmids and human cells. Nevertheless, a couple of observations might support our aim to investigate this phenomenon further: I) As mentioned above, it is generally accepted that bacterial metabolomes in the gut can influence the pathogenesis of CRC. Also, it is known that plasmids can provide a whole spectrum of additional properties to bacteria. II) Gut is a specific space where the massive intake of various molecules is localized and optimized. III) Some human tumorigenic viruses like HPV, KSHV, or EBV replicate themselves in a plasmid-like manner in human cells (28); thus, the replication of bacterial plasmids even in human cells is not excluded. IV) Tumor-inducing plasmids (Ti-plasmids) are relatively common in the plant realm (29, 30).

The aim of this preliminary study is to provide a proof of concept that random plasmids from gut bacteria can be isolated and amplified by means of the RCA method. Our previous work has shown that such amplified products (obtained from other sources) can be further processed, digested, cleaved, cloned, and sequenced (31). However, the collection of plasmid sequencing data and their comparison between CRC patients and healthy controls would be just a first step to elucidate this possibly existing interaction network.

MATERIAL AND METHODS

The design of workflow for semi-selective isolation and amplification of random circular sequences from intestinal flora is illustrated in Figure 1.

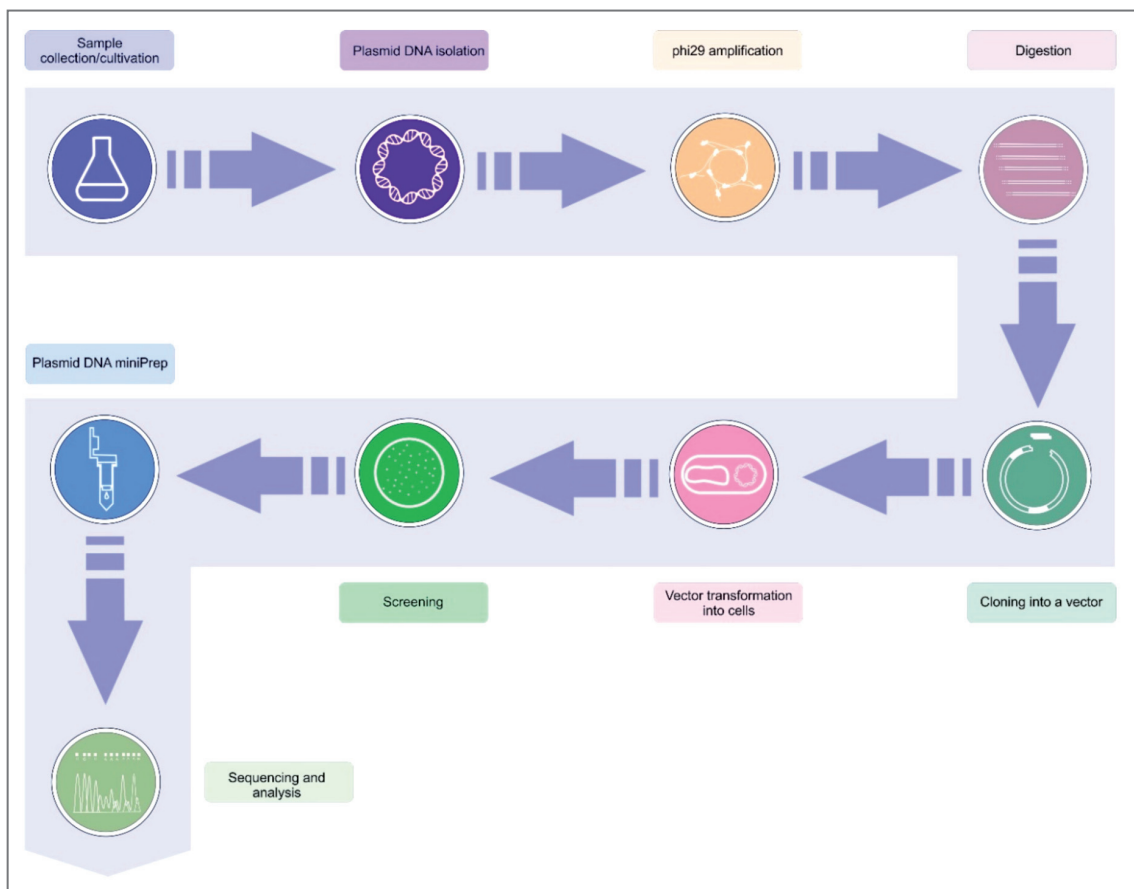


Fig. 1 Proposed workflow for the isolation of plasmid sequences from column bacterial flora.

Sample cultivation:

Stool samples were cultivated in LB (Invitrogen) without antibiotics at 37 °C overnight, shaking 200 RPM. Plasmid DNA isolation was performed with PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's protocol.

Isolation of circular DNA from gut microflora:

The mixture of the isolated plasmids DNA served as a template for *Phi29* DNA polymerase (New England Biolabs). Exo-resistant random hexamers (Thermo Scientific™) were used as primers. First, the mixture of *Phi29* polymerase buffer, hexamers, and DNA in water was incubated at 95 °C for 3 minutes, then cooled down to 4°C to allow the separation of DNA double-strand and primer annealing. After primer annealing, the mixture of thermolabile *Phi29* polymerase, *Phi29* polymerase buffer, another half of hexamers, BSA, and dNTPs diluted in water was added and incubated at 30 °C for 18 hours; then the product was heat inactivated at 65 °C for 10 minutes and cooled down to 4 °C.

Cleavage of RCA product:

Subsequent cleavage of the products was performed using restriction endonucleases (New England Biolabs) with belonging buffers at 37 °C for 1 hour. The following enzymes from pUC19 MCS were selected and used: *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, and *Xba*I.

Electrophoretic separation of digested DNA fragments:

Electrophoresis allows the separation of digested DNA fragments according to size. The 1.5% gel was prepared from Certified PCR Low-melt Agarose (Bio-Rad) and TAE solution (Thermo Scientific). Midori Green Advance (NIPPON Genetics) was used for DNA staining.

Isolation of DNA fragments from the gel:

Cleaved DNA fragments were cut out from the gel and isolated using NucleoSpin Gel and PCR Clean up kit (Macherey-Nagel) according to the manufacturer's protocol.

Cloning of the isolated fragments into the vector pUC19:

Isolated cleaved-out fragments were cloned into the pUC19, opened with the respective restriction enzyme, and dephosphorylated with FastAP (Thermo Scientific) using Rapid Ligation Kit (Thermo Scientific).

Transformation of ligation products into competent bacteria:

Further, ligation mixtures were transformed into DH5 α competent bacteria (Invitrogen) plating on agar plates supplemented with ampicillin (AMP) (Gibco). Colonies were grown overnight, at 37 °C, in the bacterial incubator.

PCR screening:

Bacterial colonies were screened for positive clones using Go Taq G2 Green Master Mix (Promega), with in-house designed primers pUC19-seq-F and R (5' TGGAATTGTGAGCG-GATAAC 3' and 5' ATTAAGTTGGGTAACGCCAG 3') (Integrated DNA Technologies). After electrophoretic separation, clones producing fragments of the expected size were selected.

Miniprep isolation:

Selected colonies were grown in LB supplemented with AMP, at 37 °C overnight, shaking 200 RPM. Again, plasmid DNA isolations were performed with PureLink™ Quick Plasmid Miniprep Kit, during the procedure identical to sample processing.

Sequencing:

Isolated DNA sequences were sequenced using an in-house sequencing assay and the same primers that were used for the PCR screening.

Data analysis:

For the archiving, identification, comparison, and analysis, free software, namely: Blast (National Library of Medicine), SerialCloner 2-6-1, and Chromas 1.45, were used.

RESULTS

In order to test the suitability of the designed workflow, we performed sample collection, cultivation, isolation of small circular bacterial DNAs, RCA isothermal amplification with random primers, and electrophoretic analysis. Fig. 2 shows the outcome of the procedure. The line 1. with negative control shows no sign of massive DNA production. On the contrary, line 2. demonstrates massive amplification in the RCA reaction with the template DNA isolated from bacteria. Most DNA products are of the size of several 10 kbp; a smaller fraction of amplified DNA wasn't able to leave the well due to its size. Positive control represented by plasmid pUC19 contained amplified DNA, of which the majority gave band between 1500 and 5000 bps after treatment with EcoRI, corresponding with pUC19 size of 2686 bp.

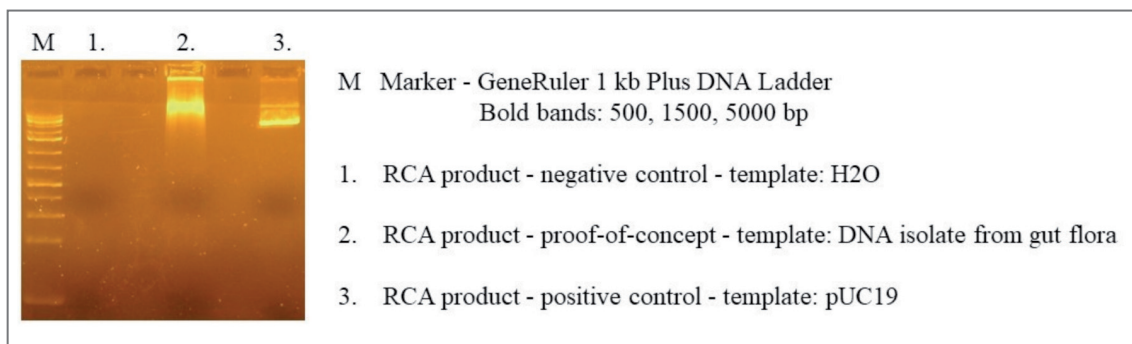


Fig. 2 Results of RCA amplification with the presumably circular DNA template isolated from gut bacteria visualized on the electrophoretic gel

We have also optimized and trouble-shot further downstream steps of the protocol. However, this part was already tested and published previously with the template obtained from bovine serum (31). (Data not shown.)

DISCUSSION

The aim of this preliminary study was to test the protocol modified for the isolation of DNA sequences, preferably from plasmids of gut bacteria. Several issues deserve consideration. For example, the cultivation of a sample can influence the ratio of particular bacterial species. The ones that are hard to promote in LB media would be underrepresented. Thus, direct isolation from a sufficient amount of sample shall be considered.

The concept of this project is based on these specific facts: I) *Phi29* polymerase is known for an effective amplification of circular molecules; therefore, plasmids, circular viral genomes, and other circular molecules are multiplied preferably (32). II) Strand displacement activity, high fidelity, and extreme processivity of *Phi29* polymerase allow a highly accurate generation of large fragments during the isothermal reaction. Thus, the product is composed of multiple copies of circular template sequences linked in linear DNA fragment large up to 100 kbp (33). III) The random sequence of six nucleotides can be expected to repeat every 4096 nucleotides (4⁶) in unknown DNA. Most of the restriction endonucleases recognize six nucleotides long palindrome. Thus, it is reasonable to speculate that at least some from the set of enzymes recognizing six nucleotide sequences will cleave unknown DNA, resulting in the fragment and/or fragments long several hundreds or thousands of bps. Naturally, the increased number of enzymes used increases the chance of capturing and identifying more sequences, thus expanding the database of sequences.

For the sake of simplicity, we used the pUC19 vector with selected restriction enzymes; however, any suitable vector with an appropriate set of enzymes could be used.

Provided that the isolation of whole circular sequence is needed or desired, long PCR with back-to-back primers can be designed based on the partial sequence.

Using this approach, valuable data with the potential to describe the differences between the plasmids of bacteria in healthy controls and CRC patients or even elucidate the eventual role of plasmids in the etiology of CRC can be gained. Further investigation is needed. This proof of concept shall serve as a basis for the grant application.

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Received: 20.02.2024

Accepted: 27.02.2024