Which bacterial toxins are worthy of validation as markers in colorectal cancer screening? A critical review

Kristina Mezerova¹, Vladislav Raclavsky¹, Lubomir Stary²

Appropriate screening of early asymptomatic cases can reduce the disease burden and mortality rate of sporadic colorectal cancer (CRC) significantly. Currently, fecal occult blood testing (FOBT) is able to detect up to 80% of asymptomatic cases in the population aged 50+. Therefore, there is still a demand for new screening tests that would complement FOBT, mainly by detecting at least a part of the FOBT-negative CRC and adenoma cases, or possibly by identifying person at increased risk of sporadic CRC in order to offer them tailored follow-up. Among the potential markers studied, our knowledge has advanced at most in toxigenic gram-negative bacteria. In this review, we assess their potential critically and recommend those best suited for prospective evaluation of their true ability to increase the sensitivity of FOBT when combined during general population screening. In our opinion, colibactin and Bacteroides fragilis toxin are the best candidates, possibly complemented by the cytotoxic necrotizing factor (CNF).

Key words: colorectal cancer, enterotoxin, colibactin, cytolethal distending toxin, cytotoxic necrotizing factor, cycle inhibiting factor, Bacteroides fragilis toxin

Received: April 1, 2021; Revised: August 18, 2021; Accepted: August 25, 2021; Available online: November 5, 2021
https://doi.org/10.5507/bp.2021.054
© 2021 The Authors; https://creativecommons.org/licenses/by/4.0/

¹Department of Microbiology, Faculty of Medicine & Dentistry, Palacky University Olomouc, Czech Republic
²Department of Surgery I, University Hospital Olomouc, Czech Republic
Corresponding author: Lubomir Stary, lubomir.stary@fnol.cz

INTRODUCTION

In 2020, colorectal cancer showed the 3rd highest incidence in men (23.4 new cases per 100,000) and the 2nd highest in women (16.2) worldwide. In the Czech Republic, its incidence was even higher – 38.0 in men and 24.6 in women, respectively¹. Czech male patients are among the 3 highest CRC risk populations in Europe². Although the majority of the patients with CRC were diagnosed in early stages, the incidence of CRC increased significantly in the Czech Republic from 1982 to 2002 (ref.³). For brief comparison of the Czech population CRC incidence and mortality to other countries see Table 1. Due to its typically slow progression, most cases of CRC can be successfully treated, provided they are diagnosed in an early stage. This can best be achieved by appropriate screening strategy. Smaller part of cases, termed hereditary CRC (1-5%), typically develop in younger people, whereas most of the cases, termed sporadic CRC, develop after the age of 50. Therefore, if CRC is present in family history of a person, screening should be performed at younger ages and its frequency should be tailored according to individual risk assessment. In the general population, screening of sporadic CRC is recommended after the age of 50.

Due to the accessibility of colon to endoscopy, colonoscopy is the best primary screening option. However, adherence to colonoscopy is not high, because of worrying about pain, complications and discomfort. Because most of CRC lesions release small amount of blood, which can be detected in a stool sample, a fecal occult blood test (FOBT) represents an alternative to primary screening colonoscopy. Depending upon the performance of the particular testing set, CRC or adenoma underlies 10-30% of its positive results⁴,⁵, the rest being caused by other sources of bleeding. A positive FOBT result increases the adherence to the following diagnostic colonoscopy distinctly – in the Czech Republic, 36 086 primary screening colonoscopies were performed in 2006-2015, as compared to 154 996 colonoscopies following a positive FOBT (ref.⁶).

Table 1. Estimated age-standardized rates of CRC incidence and mortality in 2020 - compared to selected other countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Incidence</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Czech Republic</td>
<td>33.7</td>
<td>12.3</td>
</tr>
<tr>
<td>Neighboring or close countries</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>25.8</td>
<td>9.9</td>
</tr>
<tr>
<td>Poland</td>
<td>30.5</td>
<td>16.1</td>
</tr>
<tr>
<td>Slovakia*</td>
<td>43.9</td>
<td>21.0</td>
</tr>
<tr>
<td>Austria**</td>
<td>21.0</td>
<td>8.7</td>
</tr>
<tr>
<td>Hungary***</td>
<td>45.3</td>
<td>20.2</td>
</tr>
<tr>
<td>Other countries</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO Europe</td>
<td>28.8</td>
<td>12.0</td>
</tr>
<tr>
<td>USA</td>
<td>25.6</td>
<td>8.0</td>
</tr>
<tr>
<td>Japan</td>
<td>38.5</td>
<td>11.6</td>
</tr>
<tr>
<td>China</td>
<td>23.9</td>
<td>12.0</td>
</tr>
<tr>
<td>World</td>
<td>19.5</td>
<td>9.0</td>
</tr>
</tbody>
</table>

*second highest incidence in Europe
**lowest incidence in Europe
*** highest incidence in Europe
Importantly, FOBT testing enables detection of around 80% of new asymptomatic CRC cases and 50-60% of adenomatous polyps. However, there is still a demand for new screening tests that would complement FOBT, mainly by detecting at least a part of the FOBT-negative CRC and adenoma cases, or possibly by identifying person at increased risk of sporadic CRC in order to offer them more frequent periodic screening. Detection of tumor-specific genetic material, namely detection of microRNA and DNA methylation, represents one group of promising markers\(^7\), which is, however, out of the scope of this review. Because colonic microbiota is widely accepted to play important role in the development of sporadic CRC, it represents another source of potential CRC screening marker. Unfortunately, despite the recent advances of the techniques of gut microbiome analysis, their standardization is still not sufficient to bring them closer to routine use. Therefore, targeted detection of particular promising microbial markers represents a more feasible approach in the near future. Among these, toxicigenic gram-negative rods are the best studied and most promising candidates so far. In this review, we assess their potential critically and recommend those best suited.

**COLIBACTIN**

Recently, lot of studies were focused on colibactin, a potentially CRC-associated genotoxin. Nougayrède et al.\(^1\) discovered this hybrid peptide-polyketide genotoxin in the B2 phylogroup *E. coli* and demonstrated its ability to induce DNA double-strand breaks (DSBs) *in vivo*.

**Mechanism of action**

Colibactin is encoded by genes present in the 54 kb polyketide synthase (*pks*) pathogenicity island. The *pks* island includes *clbA*-S genes encoding 3 non-ribosomal peptides (C1bH, C1bJ, C1bN), 3 polyketide–synthases (PKS: C1bC, C1bH, C1bO), 2 hybrid molecules (C1bB, C1bK) and 9 accessory and tailoring enzymes\(^8\).

Mammalian cells infected by a low dose of colibactin producing *E. coli* showed a reversible damage of DNA followed by incomplete reparation, and thus resulting in DNA-damaged cells after cell division. Colibactin producers also induced anaphase bridging, chromosome aberrations, aneuploidy and tetraploidy in the infected cells. Moreover, increase in gene mutation rate and anchorage-independent colony formation were observed in the infected cells\(^8\).

Molecular pathogenesis of the colibactin-mediated DNA damage was clarified by Li et al.\(^9\), who characterized its mature metabolite, colibactin-645. Namely, this showed an unusual heterocycle-fused macrocyclic structure that mediated copper binding and reduction leading to DNA DSBs *in vitro*.

**Supporting evidence**

Colibactin action has been explored by many scientists so far and recent studies contributed significantly to our understanding on how it promotes colon tumor formation. With the help of *in silico* modelling it was established that the regions affected by colibactin-645-mediated DSBs harbor AT-rich penta/hexameric sequence motifs with unique characteristics. Subsequently, these colibactin damage motifs (CDMs) were found to be enriched in mutations in cancer genomes, providing another direct proof of colibactin genotoxicity\(^11\).

Despite the fact that colibactin molecule is unstable and many attempts for its isolation by traditional approaches failed, its structure came to light in 2019. Due to combination of chemical synthesis, mass spectrometry, genetics and bioinformatic techniques, the team led by Mengzhao Xue et al.\(^12\) uncovered the mystery of this molecule. This was crucial for further understanding of genotoxic and cytotoxic mechanisms of colibactin action. Wilson et al.\(^13\) were able to compare the DNA adducts present in cells infected by *pks*\(^+\) *E. coli* to those present in cells infected by a mutant strain to DNA from cells infected by a strain missing the *pks* island. This experiment provided direct evidence of the colibactin ability to alkylate DNA *in vivo*. In addition, the human intestinal organoids exposed to *pks*\(^+\) *E. coli* showed a mutational signature resulting from single base substitutions (SBS) and deletions at adenine-enriched motifs\(^14\). This study amended the previously obtained results of Kusibab et al.\(^11\) and Wilson et al.\(^13\), by describing the formation of adenine-colibactin adducts and DNA interstrand cross-links\(^14\). As Pleguezuelos-Manzano et al. suggested, the DNA crosslinks formed between two adenosines could induce the DSBs, nucleotide excision repair and translesion synthesis leading to above mentioned SBS\(^{pks}\) and deletions, termed ID-\(pks\)\(^14\). As a recent review proposed, this study directly links the mutations associated with higher risk of CRC development with colibactin exposure\(^15\).

**Potential for screening**

Due to its confirmed genotoxicity, colibactin has been proposed to serve as a noninvasive CRC marker that can be detected in an easily available stool sample or a rectal swab. Many studies have analyzed the prevalence of colibactin in CRC patient samples versus healthy controls in order to verify its usefulness as a CRC marker. As one example for all, the gene *clbA* was detected by qPCR in stool samples of patients invited for colonoscopy after presenting with symptoms from the large bowel. Positive qPCR results was obtained 56.4% of CRC patients versus 31.3% of dysplasia and 18.5% of negative patients\(^16\).

**Open questions and limitations**

Nowadays there is no doubt that the genotoxin colibactin is able to harm the cells so much that it can facilitate transformation of healthy mucosa into carcinomas\(^17\). However, several authors claim that colibactin producers are present also in samples from healthy people\(^18\)\(^20\), even in samples coming from healthy newborns\(^18\)\(^22\). It should however be noted that the harmful potential of *pks*\(^+\) *E. coli* may be rather different in younger versus older people. It is quite conceivable that *pks*\(^+\) *E. coli* is unable to induce CRC de novo, but it does facilitate the progression of pre-cancerous lesions into cancer. In such case, its presence
would be linked to CRC only in people aged 50+, where the sporadic CRC develops typically. Be it as it may, the promising results of existing studies have yet to be proved in prospective screening studies.

Furthermore, number of studies published on colibactin in CRC so far relied on detection of the clb genes\cite{18,23-30}. In such detection settings, the open questions are whether the genotoxin is present in its active form in the intestinal tissue and what triggers its expression. Inflammation was demonstrated to be required for expression of pkS-associated genes in a mice model of CRC-tumorigenesis\cite{31}; another study also supports the hypothesis that a hit such as an APC mutation is needed for the expression of colibactin genes. Moreover, the extent of expression is associated with the CRC stage too; the latter the CRC stage is, the higher the expression\cite{32}.

Unfortunately, the data obtained by Pleguezuelos-Manzano et al. suggest that E. coli Nissle 1917 (EcN) could induce the characteristic mutational signature also observed in human intestinal organoids exposed to colibactin, thus questioning its safe therapeutic use\cite{14}. In an attempt to solve this problem, Massip et al. succeeded to decouple the antibacterial activity of EcN from its genotoxic activity. It should be noted that a gene clBP coding for colibactin peptidase was found to be required not only for an activation of colibactin, but also for the antibacterial activity of EcN. This antibacterial activity is mediated by two siderophore-microcins (Mcc). Massip et al. firstly demonstrated ClbP to be involved both in colibactin activation and in the antibacterial activity of EcN. Secondly, in a series of clbP mutants, they identified a single amino acid substitution that inactivates the genotoxic, but maintains the antibacterial activity of EcN, whereas a complete deletion of the clbP gene resulted in its loss. This indicates an involvement of the ClbP transmembrane domain in Mcc biosynthesis or secretion, and presumable co-evolution of the pks island and the siderophore-Mcc biosynthesis pathway\cite{33}. More importantly, it opens the way to the safe use of a modified EcN in clinical practice.

Yet another study focused on the potential of pkS+ EcN in tumor targeting therapy\cite{34}. EcN could be used as a carrier delivering cytotoxic compounds directly into CRC tumors since it has been shown to decouple the tumor tissue. The cytotoxic compound-colibactin complex could be activated by over-expression of mtaA encoding myxothiazole gene cluster, a key enzyme for colibactin pathway\cite{34}. Undoubtedly, these promising results encourage further explorations in this direction. Doubts about the toxigenic E. coli being a cause or effect of CRC were recently summarized and framed by Wassenaar\cite{35}, who argue that cell and animal models proving its carcinogenic action are rather artificial; the clinical data on associations of toxigenic E. coli and CRC can equivalently be explained as enhanced and prolonged colonization resulting from the disease itself. This alternative hypothesis also proposes colibactin not to act as a carcinogen, which is not evolutionary meaningful, but rather as a bacteriocin\cite{35}.

**Standpoint on suitability and feasibility for screening purposes**

Altogether, in case colibactin really does promote CRC, it looks like its expression and thus true pro-oncogenic action relies upon numbers of processes, preferentially the inflammation, genetic predisposition and the stage of CRC. Colibactin-positive E. coli itself is not necessarily a CRC promoting factor, it could be just a harmless commensal bacterium in those hosts who do not harbor or are not exposed to other CRC-promoting factors. From this point of view, the colibactin would rather represent a co-factor promoting CRC development in predisposed host and/or facilitate its progression.

However, this does not necessarily preclude its suitability for screening purposes. As aptly remarked by Wassenaar who questioned the true CRC-promoting action of pkS+ E. coli in human colon: ‘Patients suffering from CRC are not helped by work demonstrating that they can, at least in part, blame their bacteria for the disease’ (ref.\cite{35}). Despite the bluntness of this statement, it also means if a bacterium can be reliably associated with asymptomatic presence of cancer in clinical studies, it is worth of exploring its potential as biomarker of the disease, irrespective of being possibly just a consequence, not cause of the disease.

On the other hand, it should also be noted that answering the question of cause or effect has its important practical implications as well – in case causative or promoting role of colibactin or other E. coli toxins in CRC development would be proved, identification of its sources in human diet and/or environment may represent an attractive target for primary prevention of CRC.

Be it as it may, the time has come for prospective studies of colibactin gene detection on wide real population eligible for FOBT, best performed from the same sample along with FOBT to establish its true potential to contribute to better performance of current CRC screening strategies. Although expression analysis from targeted sampling would be the best option in the context of the conditional association with CRC outlined above, this is not well suited for routine screening purposes. Thus, despite the limited specificity of mere gene detection, it can still be useful for screening given it can significantly increase the sensitivity or specificity of the currently widely available FOBT.

**CYTOLETHAL DISTENDING TOXIN (CDT)**

Different types of toxins with similar cytotoxic activity are summed under the term CDT. A heat labile cytotoxic toxin produced by E. coli and Campylobacter sp. isolates was firstly described by Johnson and Lior in 1988. They demonstrated the cytotoxicity of the producing strains on Chinese hamster ovary cells, Vero, Hela and Hep-2 cells. The CDT-producing strains caused progressive cell distension, however they were neither invasive, nor hemolytic\cite{36,37}.
Mechanism of action

At the molecular level, the CDT is composed of three proteins, namely CdtA, CdtB and CdtC, that vary in sequence homology across bacterial species. The cdtB gene coding for the CdtB subunit was characterized as the most conserved and the largest one with 819 bp length. The cdtA and cdtC subunits comprise 711 and 570 bp, respectively. The three slightly overlapping cdtA-C genes are located on a chromosome; on the contrary to the plasmid-harbored cdt-III allelic cluster. This cdt-III allele is located on a plasmid together with the cnf2 gene encoding cytotoxic necrotizing factor (CNF) (ref.39).

Although there are five variants of CDT alleles, expression of all three cdtA-C genes is always required for CDT cytotoxic activity38,40. The CdtA and CdtC subunits are needed for translocation of the CdtB subunit into a host cell, where the genotoxin can promote its harmful action. The CdtB subunit, which is necessary for toxin induced DNA damage41, is homologous to mammalian DNaseI and hence demonstrates nuclease activity in vivo and in vitro, resulting in DNA fragmentation and reversible cell cycle arrest38–44. In addition, CDT is also able to irreversibly block cells at the G1/S/G2/M phase of their cell cycle depending on the cell type38,45–47. Moreover, CDT was described to be able to induce DSBs in non-proliferating cells as well44,45. The formation of DSBs activates a DNA damage responses avoiding replication, which leads to genetic instability favoring tumor promotion46,49.

Supporting evidence

The genotoxic activity of CDT was demonstrated not only in vitro, but also in vivo39,50,51. In vitro studies confirmed its harmful effect on rat fibroblasts, rat small intestine epithelial cells and human colon cancer cell lines, including increased frequency of mutations, chromosomal aberration and cell cycle disruption39,52. Irreversible megalocytosis caused by CDT-positive strains was observed in HeLa cells as well46. Moreover, in a recent study by He et al.52, the CDT producing Campylobacter jejuni was proved to be tumorigenic; mice infected by a CDT-producing human C. jejuni clinical isolate displayed an increased number of tumors compared to controls. Although there was no difference in the inflammation between the infected and control animals histologically, an increase of proliferating cell nuclear antigen was observed in the CDT-positive group. Moreover, the nuclear β-catenin was overrepresented in colonic mucosa; remarkably, its overexpression – driven by the down-regulation of the well-known tumor suppressor called adenomatous polyposis coli (APC) – is associated with CRC (ref.22). Even more insight into the true role of CDT in CRC was brought by the study of Graillot et al.51, who exposed three isogenic cell lines to E. coli CDT. Whereas precancerous APC- and p53-deficient cells showed impaired DNA damage response after CDT exposure, cells expressing oncogenic KRAS were more resistant, indicating that CDT does not initiate CRC by itself, but may have promoting effect in premalignant colonic lesions53.

Potential for screening

As it was confirmed in many studies, the CDT cyclomodulin produced by E. coli strains was detected more frequently in tumors compared to healthy tissue35. In contrast to colibactin, however, the prevalence of CDT-positive E. coli isolates is much lower35. Therefore, this cyclomodulin alone can hardly be used as a sensitive marker of CRC.

Open questions and limitations

Interestingly, increased frequency of E. coli harboring the CDT gene was observed in colonic mucosa of CRC patients with stages II, III or IV compared to the early stage I (ref.22). In case such direct relationship between CDT-positivity and CRC staging would be confirmed in large patient cohorts, it may serve as a marker of advanced CRC before surgery. However, in most cases, screening or diagnostic colonoscopy should be much more informative. More important, lower frequency of CDT in early stages of CRC should rather disqualify it as an early screening marker.

Standpoint on suitability and feasibility for screening purposes

Taken together, CDT detection in fecal samples is for sure feasible, but its true potential to contribute to CRC screening sensitivity and specificity remains to be established. Although it cannot be excluded that its detection may increase the sensitivity of screening in combined assays with other markers, we consider such chance to be rather low.

CYTOTOXIC NECROTIZING FACTOR (CNF)

This cyclomodulin was firstly described in cytotoxic E. coli strains causing morphological alterations in HeLa cells and necrosis in rabbit skin34. CNF is a cyclomodulin interfering with cytokinesis, resulting in formation of giant flattened multinucleated cells in several cell lines35. It is the first toxic factor described to enhance polymerization of actin leading to reorganization of microfilaments and microtubules and construction of actin complex networks in the perinuclear region of the cell35. Two types of this cyclomodulin are encoded by two different genes – the cnf1 gene was detected only at chromosome, whereas the cnf2 is plasmid-encoded. Apart from E. coli, Yersinia pseudotuberculosis is also able to produce this toxin (CNFα) that enables it to modulate inflammatory response in infected cells37.

Mechanism of action

CNF is a dermonecrotic toxin that permanently activates the p21 Rho GTPase (ref.38). This GTPase is involved in actin cytoskeleton formation as a member of a concerted signaling network. Among others, this GTPase is also crucial for cell spreading, the formation of adhesion plaques and other actin-dependent events39,59.

CNF harbors 2 domains: The N-terminal delivery domain and the C-terminal deamidase domain. In the course
of endocytosis, the toxin is transported into a target cell where the deamidase domain is cleaved off and delivered into cytoplasm60-64. This C-terminal domain then deamidates Rho, Rac1 and CDC42 proteins leading to permanent p21 Rho GTPase activity by blocking its hydrolysis activity. Stimulated GTPase induces polymerization of actin resulting in formation of stress fibers, lamellipodia, filopodia and other actin-related phenomena62-65.

Open questions and limitations

Despite the fact that the mechanism of tumorigenic action of CNF1 was described in colon cancer cells, the prevalence of CNF-positive isolates in CRC samples is not as high as in the case of colibactin positive isolates. There is a need for further investigation on the potential of CNF detection in increasing the sensitivity or specificity of colibactin detection in CRC screening

CYCLE INHIBITING FACTOR (CIF)

Cyclomodulin Cif has an ability to arrest a cell cycle in G2 phase without inducing DNA damage. Although the effect of Cif is very similar to CDT; the Cif toxin does not cause DSBs and, hence, this cyclomodulin cannot be considered as a genotoxin. This is an important difference compared to the toxins described above, action of which relies on DNA damage pathways. Nougayrede et al.74 first described the enteropathogenic E. coli (EPEC) with type III secretion system (TTSS) in 1999, inducing the rearrangement of host cell cytoskeleton 74. The toxin called Cif and identified only later was also found in enterohaemorrhagic E. coli (EHEC), harboring the same TTSS secretion system and causing similar pathologic effects in infected cells75.

Mechanism of action

The translocated effector molecule Cif is able to trigger a cytopathic effect in the infected HeLa cells resulting in inhibition of G2/M phase transition and in formation of actin stress fibers76,77. The arrested cells accumulate in G2 phase without inducing DNA damage. Although the effect of Cif is very similar to CDT; the Cif toxin does not cause DSBS and, hence, this cyclomodulin cannot be considered as a genotoxin. This is an important difference compared to the toxins described above, action of which relies on DNA damage pathways. Nougayrede et al.74 first described the enteropathogenic E. coli (EPEC) with type III secretion system (TTSS) in 1999, inducing the rearrangement of host cell cytoskeleton 74. The toxin called Cif and identified only later was also found in enterohaemorrhagic E. coli (EHEC), harboring the same TTSS secretion system and causing similar pathologic effects in infected cells75.

Supporting evidence

CNF1 has been associated with human clinical isolates of uropathogenic (UPEC) and enteropathogenic E. coli (EPEC) (ref.66,67). The occurrence of a group of genes including cnf1 and present in a pathogenicity island of UPEC was detected also in a subgroup of meningitis-associated strains, suggesting that CNF1 may be one of the virulence factors enabling meningeal invasion. A gene encoding hemolysin was also detected in the analyzed and present in a pathogenicity island cnf1 genes27. Earlier, the CNF1 toxin production is linked with hemolytic E. coli phenotype. This association was also confirmed by Falbo et al.68 who observed co-occurrence of the cnf1 and hly genes in the same DNA region69. On the contrary to CNF1, the CNF2 is not associated with hemolytic phenotype, however is more lethal in mouse intraperitoneal tests and was identified in both intraintestinal and extraintestinal human infections70,71.

Genotoxic activity of CNF in vitro and in vivo was demonstrated in a number of studies72-78,79. In laboratory rats, the study by Kurnick et al.70 confirmed co-occurrence of CNF, colibactin and CDT in isolates of pathogenic E. coli. The cytopathic effects of CNF and CDT were also confirmed in cytotoxicity assays26. Similar results were demonstrated in the study by Fabian et al.71, where megalocytosis and cell death were observed in HeLa cell lines infected by pathogenic strains E. coli harboring CNF1 genes72.

In a recent study, Zhang et al.72 demonstrated the genotoxic property of CNF in colon cancer cells, proving its carcinogenic effect via driving the treated cells into irreversible senescence. The upregulation of p53, p21, p16 and β-galactosidase activity leading to genomic instability was observed, as well as aneuploidy of infected cells. The survival of CNF1-intoxicated cells remained undetermined until Zhang et al.72 revealed the mechanism of reprogramming the cells to survive the intoxication. It turned out that the key for cell survival is the ability of multinucleated cells to enter another cell division cycle instead of proliferation arrest. As multinucleated polyploid cells are formed in response to CNF action, asymmetric cell division follows during depolyploidization, which results in genomic unstable aneuploid daughter cells. Notably, aneuploid cells are one of the most common phenomena associated with tumors72.

Potential for screening

High prevalence of CNF-producing E. coli in cancer patients was demonstrated in several studies implicating its importance in CRC pathologies80,81,82,83,84. In the study by Hilali et al.72, the E. coli isolates harboring cnf1 genes were detected in blood from cancer patients. CNF-positive E. coli was isolated more frequently from biopsies of colon tumors compared to diverticulosis patients (P<0.02), and, in diverticulosis patients, the samples free of E. coli were more prevalent (19.4%) compared to samples from colon tumors (2.6 %) (ref.33).
indicating that Cif affects more than these two proteins involved in the cell cycle machinery. The Cif crystal structure was revealed by Hsu et al., suggesting structural homology to the *Pseudomonas syringae* AvrPhB enzyme. A core anti-parallel β-sheet and an N-terminal helix packaging against the β-strands are always present in this enzyme family, which includes cysteine proteases, transglutaminases, and acetyltransferases. The Cif protein contains a catalytic triad composed of nucleophile Cys109 on the N-terminus of the α-helix, His165 located on the N-terminus of the β-strand, and the third residue Gln185 on the C-terminal end of the β-strand. This catalytic triad is crucial for induction of cell cycle arrest, enlargement of infected cells, and for their cytoskeletal rearrangement. Importance of the three domains was tested by creating point mutations in the triad and infecting HeLa cells by these mutants. The infected cell lines did not display any perturbations of cell cycle progression.

Supporting evidence

In addition to HeLa cells, the cytostatic effect of Cif was also tested in intestinal epithelial cells (IEC-6). This cell line simulates natural environment of EPEC infection and is hence ideal for studying the fate of Cif-infected cells. Infection of IEC-6 by Cif-positive EPEC strain resulted in apoptosis 48 hours later. This delayed apoptosis is in accordance with presumed action through inhibition of the proteasome degradation pathway.

Potential for screening

Although the cytotoxicity of Cif was proved by a number of studies, the prevalence of Cif-positive EPEC or EHEC in clinical samples is rather low compared to *coli*-ber of studies, the prevalence of Cif-positive EPEC or CNF-positive EPEC of the proteasome degradation pathway. Notably, regulated Cif. This cyclomodulin deamidates NEDD8, leading to inhibition of cell proliferation and survival, indicating promising potential in colon cancer therapy.

Standpoint on suitability and feasibility for screening purposes

Unfortunately, both its low prevalence and its possible ambiguous role in normal and cancer cells seem to disqualify Cif from being useful for CRC screening in the context of current knowledge.

**BACTEROIDES FRAGILIS TOXIN (BFT)**

*B. fragilis* is a strictly anaerobic bacterial species attracting researcher’s attention as a producer of the potentially carcinogenic BFT (ref. 84). The subgroup of *B. fragilis* producing this metalloprotease protein toxin is called enterotoxigenic *B. fragilis* (ETBF). ETBF was firstly associated with diarrheal disease in lambs and it was also detected in extraintestinal infections later.

Mechanism of action

Biologic activity of BFT was firstly demonstrated on the model of human colonic carcinoma epithelial cells (HT29/C1). This was the first culture tissue assay proving the toxic activity of BFT, which was reflected in the loss of cell-to-cell attachments, swelling and other morphological changes of treated cells. As it was clarified later, BFT binds to a specific membrane receptor in the intestinal epithelial cells. Its attachment to the cells leads to proteolytic cleavage of E-cadherin. E-cadherin is associated with the T-cell factor dependent nuclear signaling protein β-catenin. Following the loss of E-cadherin, the β-catenin is released and its nuclear localization takes place leading to upregulation of c-Myc transcription and translation. As a result, persistent cellular proliferation is induced.

Further studies of the BTF’s enterotoxic activity in ApcMin mice colonized by ETBF revealed induction of a cascade of pro-carcinogenic inflammatory reactions through IL-17-dependent NF-κB activation and Stat3 signaling in distal colon epithelial cells (CEC), triggering the myeloid cell-dependent distal colon tumorigenesis.

Even though the pro-inflammatory and pro-carcinogenic effect of BFT was confirmed, the exact mechanism by which BFT stimulates the pro-carcinogenic signaling relay is still discussed. Recently, Allen et al. explained the tumorigenic potential of BFT by induction of epigenetic changes in CEC correlated with proliferation of tumor cells. According to this study, chromatin accessibility is immensely affected across the genome by BFT, including transcription factor binding sites and resulting in upregulation of transcription factor motifs such as JUND, JDP or FOSL1, which are part of mitogen-activated protein kinase (MAPK) pathways modulated by BFT.

At the molecular level, BFT, also called fragilysin, is characterized as a 20 kD heat-labile protein toxin belonging to the metalloprotease family. Sequencing of the whole *bft* gene revealed one open reading frame (ORF) of 1191 nucleotides coding for a 397-residue holotoxin pro-
tein of 44.4 kDa. BFT is synthesized as a 379 amino acid pretoxin, which becomes a biologically active protein after crossing cytoplasmic membrane. As a result, the active toxin of 186 residues with a molecular mass of 20.7 kDa is secreted. Comparison of the known bft gene sequences revealed three regions of reduced homology. On the bases of these data, BFT can be divided into three isoforms (BFT-1, BFT-2 and Korea-BFT), all acting by cleavage of E-cadherin protein.89,95,96.

Supporting evidence

Familial adenomatous polyposis (FAP) is one of the hereditary syndromes caused by APC mutation which almost always leads to colon tumor formation. Colonic mucosa from patients with FAP was examined for tumorigenic bacteria including B. fragilis in the study of Dejea et al.97. Both B. fragilis and the bft gene were highly enriched in FAP patients’ colonic mucosa compared to healthy individuals. Furthermore, tumour-prone mice colonized with ETBF showed increased interleukin-17 in the colon and DNA damage in colonic epithelium with faster tumor onset and higher mortality.98. These data correspond to the results of Chung et al.91, suggesting the importance of the T_h17-dependent pathway in BFT induced colon carcinogenesis.91.

Potential for screening

Several studies that rely on PCR detection of the bft gene in stool support its usefulness as a CRC marker. An increased prevalence of ETBF was detected in CRC stool samples (P=0.009) compared to controls.41 Similar results (P<0.05) were reported by Hagh et al.98 later. Moreover, the bft gene presence in colonic mucosa is highest in late-stage CRC among different studies.98-100. These data suggest the association of ETBF with CRC progression, and, hence, its detection in patients would refer to higher risk of CRC diagnosis.

Open questions and limitations

Undoubtedly, the significantly increased prevalence of ETBF in colonic mucosa of CRC patients, together with the revealed mechanisms of BFT action, represents a strong evidence for its role in CRC etiology. However, possible differences in the biological and tumorigenic activity of different BFT subtypes are still matter of discussion. Presumed increased BFT-2 biological activity101 led researchers to identification of particular bft gene isoforms in stool samples from CRC patients. However, the observed prevalence of BFT isoforms in samples from CRC patients varied among different studies. The highest frequency of the bft2 subtype was found by Boleij et al.99 and Hagh et al.98, whereas Zamani et al.102 reported bft1 as the most prevalent subtype followed by bft2. Furthermore, it has also been suggested that poor sensitivity may limit the accuracy of ETBF detection in stool samples, including the detection of particular isoforms.103. Last but not least, small numbers of patients included in the studies published so far represent a limiting factor that needs to be resolved.

Table 2. Summary of most informative studies published on the toxins reviewed, including our standpoint on their suitability in CRC screening.

<table>
<thead>
<tr>
<th>Action</th>
<th>Colibactin</th>
<th>Cytotoxic necrotizing factor (CNF)</th>
<th>Cycle inhibiting factor (CIF)</th>
<th>Bacteroides fragilis toxin (BFT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>16</td>
<td>107</td>
<td>107</td>
<td>84</td>
</tr>
<tr>
<td>Number of all study subjects</td>
<td>239</td>
<td>107</td>
<td>107</td>
<td>96</td>
</tr>
<tr>
<td>Number of CRC († FAP) patients</td>
<td>39</td>
<td>38</td>
<td>38</td>
<td>56</td>
</tr>
<tr>
<td>Number of cyclomodulin -positive CRC († FAP) patients</td>
<td>22</td>
<td>21</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Sensitivity*</td>
<td>56%</td>
<td>55%</td>
<td>16%</td>
<td>38%</td>
</tr>
<tr>
<td>Specificity*</td>
<td>73%</td>
<td>61%</td>
<td>91%</td>
<td>88%</td>
</tr>
<tr>
<td>Suitable for screening</td>
<td>yes</td>
<td>no</td>
<td>conditionally yes**</td>
<td>yes</td>
</tr>
</tbody>
</table>

n.a. – data not available
* The values do not represent true values of sensitivity and specificity established by properly conducted clinical studies; they are only estimates based on published data (see appropriate Ref.).
** The potential contribution of CNF detection to increased sensitivity and/or specificity of CRC screening based on colibactin detection has to be established only.
† FAP = familial adenomatous polyposis
CONCLUSION

To conclude, we concisely summarize the data available on the toxins reviewed in Table 2, including our estimate of their potential in CRC screening. As already described above, nowadays, non-invasive screening of CRC relies on detection of occult bleeding in stool (FOBT). This enables detection of around 80% of new asymptomatic CRC cases and 50-60% of adenomatous polyps. Although none of the toxins summarized in Table 2 does reach an estimated sensitivity comparable to FOBT, it is still feasible that some of them may improve the performance of CRC screening if combined with FOBT. This may happen if toxins would either enable detection of at least a part of the FOBT-negative CRC and adenoma cases, or identify person at increased risk of sporadic CRC. These would subsequently be better motivated to undergo frequent CRC screening regularly.

Unfortunately, no data from prospective clinical studies, which would include FOBT complemented by toxin detection in parallel, have been published so far. Undoubtedly, colibactin detection is the most promising complementary test for such type of studies in near future. On the contrary, CDT and CDF are most probably of no use, whereas the potential of CNF has to be determined only. However, because both the colibactin-positive and CDF-positive isolates are typically recruited from the _E. coli_ phylogroup B2 strains, the chance of additive effect is rather low. In contrast, the _B. fragilis_ toxin (BFT) positive cases can be expected not to overlap too much with colibactin-positive cases, both because of the different mode of action and also because of production by different bacterial species. This assumption is in accordance with the detection of both colibactin and BFT in the mucosal colonic biofilms of 13 cases of familial adenomatous polyposis compared to 4 cases being positive for colibactin only and 2 cases for BFT only. Despite the small number (n=25) of cases, this unique study strongly suggests that the colibactin and BFT are the best candidates for evaluation of their potential to improve the performance of current CRC screening strategies.

Search strategy and selection criteria

Data for this article were identified by searches of PubMed using the terms “colorectal cancer”, “enterotoxin”, “Escherichia coli”, “screening” and combinations of these terms, and by following references from relevant articles. We gave preference to publications presenting larger cohorts and using sound methodology. Citations from respectable journals were given special weight. Our own experience was also included.

Acknowledgements: This work was supported by the Faculty of Medicine and Dentistry, Palacký University Olomouc (IGA_LF_2021_022), by the Ministry of Education, Youth and Sports, Czech Republic (DRO 61989592), and by the Ministry of Health, Czech Republic (DROFNOI 00098892, NV19-03-00069).

Author contributions: KM: incited the review, searched and summarised the literature for CRC-related toxins; VR: critically read and discussed the manuscript; SL: outlined the clinical related parts of the manuscript, discussed recommendations and conclusions for future validation and clinical use.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

REFERENCES


