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

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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, enterotixin, colibactin, cytolethal distending toxin, cytotoxic necrotizing factor, cycle inhibiting factor, Bacteroides fragilis toxin

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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, respectively[1]. Czech male patients are among the 3 highest CRC risk populations in Europe[2]. 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 results[4,5], 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.

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

*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, toxigenic 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. Nougayréde et al.[8] 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 (ClbH, ClbJ, ClbN), 3 polyketide–synthases (PKS: ClbC, ClbI, ClbO), 2 hybrid molecules (ClbB, ClbK) 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[9].

Molecular pathogenesis of the colibactin-mediated DNA damage was clarified by Li et al.[10], 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 crosslinks[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[21,22]. 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 precancerous 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[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[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[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[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[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[34]. EcN could be used as a carrier delivering cytotoxic compounds directly into CRC tumors since it has been demonstrated to colonize 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[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[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[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.[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 cytolethal 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[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 c*dtB* 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[38]. 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 activity[38,40]. 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 damage[41], is homologous to mammalian DNaseI and hence demonstrates nuclease activity *in vivo* and *in vitro*, resulting in DNA fragmentation and reversible cell cycle arrest[42–44]. 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 type[38,45-47]. Moreover, CDT was described to be able to induce DSBs in non-proliferating cells as well[41,45]. The formation of DSBs activates a DNA damage responses avoiding replication, which leads to genetic instability favoring tumor promotion[48,49].

Supporting evidence

The genotoxic activity of CDT was demonstrated not only *in vitro*, but also *in vivo*[39,50,51]. *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 disruption[50,52]. Irreversible megalocytosis caused by CDT-positive strains was observed in HeLa cells as well[26]. 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.[52]). 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 lesions[51].

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 tissue[53]. In contrast to colibactin, however, the prevalence of CDT-positive *E. coli* isolates is much lower[18]. Therefore, this cyclomodulin alone can hardly be used as a sensitive a 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.[32]). 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 skin[54]. CNF is a cyclomodulin interfering with cytokinesis, resulting in formation of giant flattened multinucleated cells in several cell lines[55]. 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 cell[56]. Two types of this cyclomodulin are encoded by two different genes – the *cnf*1 gene was detected only at chromosome, whereas the *cnf*2 is plasmid-encoded. Apart from *E. coli*, *Yersinia pseudotuberculosis* is also able to produce this toxin (CNF_Y) that enables it to modulate inflammatory response in infected cells[57].

Mechanism of action

CNF is a dermonecrotic toxin that permanently activates the p21 Rho GTPase (ref.[58]). 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 events[58,59].

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 cytoplasm[60,61]. This C-terminal domain then deamidates Rho, Rac1 and CDc42 proteins leading to permanent p21 Rho GTPase activity by blocking its hydrolase activity. Stimulated GTPase induces polymerization of actin resulting in formation of stress fibers, lamellipodia, filopodia and other actin-related phenomena[62-65].

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 pathogenicity island DNA region[68]. As it was described earlier, the CNF1 toxin production is linked with hemolytic *E. coli* phenotype. This association was also confirmed by Falbo et al.[69] who observed co-occurrence of the *cnf1* and *hly* genes in the same DNA region[69]. 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 infections[70,71].

Genotoxic activity of CNF *in vitro* and *in vivo* was demonstrated in a number of studies[27,28,53,70]. In laboratory rats, the study by Kurnick et al.[26] 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 assays[26]. Similar results were demonstrated in the study by Fabian et al.[27], where megalocytosis and cell death were observed in HeLa cell lines infected by pathogenic strains *E. coli* harboring CNF1 genes[27].

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 reversible 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 tumors[72].

Potential for screening

High prevalence of CNF-producing *E. coli* in cancer patients was demonstrated in several studies implicating its importance in CRC pathology[53,68,73-75]. In the study by Hilali et al.[73], 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 ($P \leq 0.02$), and, in diverticulosis patients, the samples free of *E. coli* were more prevalent (19.4%) compared to samples from colon tumors (2.6 %) (ref.[53]).

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

Standpoint on suitability and feasibility for screening purposes

Taken together, CNF detection in fecal samples is not only feasible, but also highly desirable in proof-of-concept CRC screening studies. Although, most probably, its sensitivity for screening purposes does not reach that of colibactin, it may contribute to the sensitivity a specificity of other markers in combined assays.

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 cells[75].

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 fibers[74,76]. The arrested cells accumulate an inactive phosphorylated Cdk1 molecule, indicating that Cif inhibits Cdk1 activation, resulting in blockage of cell cycle progression[75]. Moreover, depending on the cell cycle stage at which cells are being infected, Cif also induces G1 cell cycle arrest. Such infected cells display accumulation of p21 and p27 proteins, which are cyclin-dependent kinase inhibitors. Specifically, Cif inhibits a proteasome-dependent degradation of p21 and p27 without the involvement of p53 as the main transcriptional activator, indicating that Cif does not affect the synthesis pathway of p21 or p27. Nevertheless, the G1/S cell cycle arrest is not resumed by downregulation of p21 nor p27,

indicating that Cif affects more than these two proteins involved in the cell cycle machinery[77].

The Cif crystal structure was revealed by Hsu et al.[78], 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[78].

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 faith 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[79].

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 colibactin-, CDT- or CNF-positive *E. coli*. In the study by Buc et al.[53], only 6 out of a total of 116 enterobacterial isolates were harboring the *cif* gene. The Cif positive isolates were categorized to either the A or B1 phylogenetic group characterized by low virulence[53]. Similar results were obtained in the study from Salvarani et al.[80], where Cif-harbouring *E. coli* isolates were associated with the B1 phylogroup and showed a low number of additional virulence factors.

Open questions and limitations

Low prevalence of Cif/positive *E. coli* appears to be one important limitation of its usefulness in CRC screening. Furthermore, although there is no doubt about the pathologic effect if the Cif cyclomodulin, its anticancer activity has been studied recently too. As it was demonstrated earlier, the Cif protein contributes to cell cycle arrest by accumulation of p21 and p27 proteins. The accumulation of these proteins is due to inhibition of the cullin-RING-ligase (CRL) activity, which was shown to be a promising target in cancer chemotherapy. Its partner in the CRL-pathway, the ubiquitin-like protein NEDD8, shows increased activity in cancer cells and tumor-promoting activity. Therefore, drugs inhibiting NEDD8 activation are studied as promising anti-tumor agents, including Cif. This cyclomodulin deamidates NEDD8, leading to CRL-NEDD8 pathway inhibition[81,82]. Notably, regulated expression of Cif in colon cancer cell lines resulted in inhibition of cell proliferation and survival, indicating promising potential in colon cancer therapy[83].

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[85] and it was also detected in extraintestinal infections later[86].

Mechanism of action

Biologic activity of BFT was firstly demonstrated on the model of human colonic carcinoma epithelial cells ($HT29/C_1$). 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[87]. As it was clarified later, BFT binds to a specific membrane receptor in the intestinal epithelial cells[88]. Its attachment to the cells leads to proteolytic cleavage of E-cadherin[89]. 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[90]. Further studies of the BTF's enterotoxic activity in Apc^{Min} 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[91].

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.[92] 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[93,94]. 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 protoxin, 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 tumour onset and higher mortality[97]. 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[84]. Similar results (P<0.05) were reported by Haghi 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 activity[101] 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 Haghi 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.

Standpoint on suitability and feasibility for screening purposes

Once the potential technical problems with sensitivity and specificity of *bft*-gene detection assays are resolved, ETBF indisputably represents the most promising CRC risk marker suitable for validation in broader proof-of-concept screening studies, right after and best in parallel to colibactin detection.

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

	Colibactin		Cytolethal distending toxin (CDT)	Cytotoxic necrotizing factor (CNF)	Cycle inhibiting factor (CIF)	*Bacteroides fragilis* toxin (BFT)	
Action	Double-strand breaks		Double-strand breaks	Cytokinesis interference	G2-arrest	Persistent proliferation	
Reference	16	53	53	53	53	84	97
Number of all study subjects	239	107	107	107	107	96	48
Number of CRC († FAP) patients	39	38	38	38	38	56	25†
Number of cyclomodulin -positive CRC († FAP) patients	22	21	6	15	3	21	15†
Sensitivity*	56%	55%	16%	40%	8%	38%	60%
Specificity*	73%	61%	91%	73%	96%	88%	70%
Suitable for screening	yes		no	conditionally yes**	no	yes	

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 CIF are most probably of no use, whereas the potential of CNF has to be determined only. However, because both the colibactin-positive and CDT-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[97]. 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.

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