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Faster and less invasive tools to identify patients with ileal colonization by adherent-invasive *E. coli* in Crohn's disease

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Abstract

Background and Aims: The identification of Crohn's disease (CD)-associated adherent and invasive *Escherichia coli* (AIEC) is time-consuming and requires ileal biopsies. We aimed to identify a faster and less invasive methods to detect ileal colonization by AIEC in CD patients.

Methods: CD patients requiring ileo-colonoscopy were consecutively enrolled in this prospective multicenter study. Samples from saliva, serum, stools, and ileal biopsies of CD patients were collected.

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Results: Among 102 CD patients, the prevalence of AIEC on ileal biopsies was 24.5%. The abundance and global invasive ability of ileal-associated total *E. coli* were respectively ten-fold ($p = 0.0065$) and two-fold ($p = 0.0007$) higher in AIEC-positive (vs. AIEC-negative), while abundance of total *E. coli* in the feces was not correlated with AIEC status in the ileum. The best threshold of ileal total *E. coli* was 60 cfu/biopsy to detect AIEC-positive patients, with high negative predictive value (NPV) (94.1%[80.3–99.3]), while the global invasive ability (>9000 internalized bacteria) was able to detect the presence of AIEC with high positive predictive value (80.0% [55.2–100.0]). Overall, 78.1% of the AIEC + patients were colonized by two or less different AIEC strains. The level of serum anti-total *E. coli* antibodies (AEcAb) was higher in AIEC-positive patients ($p = 0.038$) with a very high negative predictive value (96.6% [89.9–100.0]) ($p = 0.038$) for a cut-off value $> 1.9 \times 10^{-3}$.
Conclusions: More than two thirds of AIEC-positive CD patients were colonized by two or less AIEC strains. While stools samples are not accurate to screen AIEC status, the AEcAb level appears to be an attractive, rapid and easier biomarker to identify patients with Crohn's disease harboring AIEC.

KEYWORDS
adherent-invasive *E. coli*, anti-*E. coli* antibodies, CEACAM6. IBD, Crohn's disease, inflammatory bowel disease

INTRODUCTION

Crohn's disease (CD) is a chronic and disabling disorder that can lead to bowel damage and altered quality of life.[1,2] CD is a multifactorial affection, resulting from an aberrant interaction between host immune system and intestinal microbiota, potentially favored by genetic and environmental factors.[3]

Among other abnormalities, the CD-associated dysbiosis is distinguished by an increase of Gram-negative species belonging to the Enterobacteriaceae family.[4] More than 20 years ago, Darfeuille-Michaud and colleagues identified and characterized a new pathovar of CD-associated *Escherichia coli*, so-called adherent and invasive *E. coli* (AIEC).[5,6] AIEC are defined by their phenotype including the following properties (all being mandatory): ability to adhere and invade the intestinal cells, to survive and to replicate within macrophages leading to increased levels of TNF-α.[5–7] AIEC colonize the ileum of approximately one third of patients with CD (ranging from 22% to 62%), compared to only 6% among healthy subjects.[6,8–11] AIEC reference strain LF82 (Lille, France) is able to adhere to the ileal enterocytes isolated from CD surgical specimens through type 1-pili variants whose expression is strongly induced in the early stages of accession to the enterocytes.[12] In vitro, FimH adhesin, which is an AIEC-related subunit of type 1-pili, recognizes glycoproteins, including the mannosylated Carcinoembryonic Antigen Related Cell Adhesion Molecule 6 (CEACAM6) receptor expressed on the surface of intestinal epithelial cells.[13] Ileal expression of this receptor is abnormally increased in CD patients.[12] In addition, AIEC bacteria have also the ability to increase the expression of CEACAM6 receptors in intestinal epithelial cell cultures suggesting that these bacteria can promote their own colonization in CD patients.[12] The accurate role of AIEC in CD genesis is still imperfectly known but the hypothesis is that these pathobionts colonized the ileum of

Key summary

1. Summarize the established knowledge on this subject
   - Adherent and invasive *E. coli* (AIEC) are a potential cause of Crohn's disease
   - Therapeutic strategies targeting AIEC are currently investigated in several clinical trials
   - The detection of AIEC requires a burdensome procedure including colonoscopy with ileal biopsies followed by tedious laboratory steps (4 to 6 weeks-long)
2. What are the significant and/or new findings of this study?
   - More than two thirds of AIEC-positive patients are colonized by two or less AIEC strains
   - We identified faster procedures for AIEC detection from ileal biopsies
   - Stools samples are not appropriate to screen patients for AIEC colonization
   - AEcAb level could be a less invasive biomarker to screen CD patients for ileal colonization by AIEC

predisposed patients and are involved to the early steps of CD development.

There are several ongoing trials assessing the efficacy of drugs targeting host-AIEC interaction that require the identification of patients carrying AIEC bacteria who could be more likely to benefit from these strategies. As no molecular biomarker have been identified so far,[14] the gold standard to characterize AIEC bacteria is a time consuming and invasive procedure (needing 4–6 weeks), requiring a colonoscopy to take ileal biopsies, followed by several tedious steps in the laboratory to verify that all the properties defining the AIEC phenotype are present. In this context, there is an unmet need for faster and less invasive procedure to identify CD patients with ileal colonization by AIEC. As blood puncture is better accepted than colonoscopy with biopsies,[15] serological marker of AIEC colonization could be of great value. Although there is currently no available data, the search for antibodies against *E. coli* could be one of these candidate marker, as microbiota-associated antigens have been observed in patients with CD.[16]

In this multicenter prospective study, we aimed to identify faster and less invasive tools to detect ileal colonization by AIEC in patients with CD.

METHODS

Ethical considerations

The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements. Written, informed consent was obtained from each patient included in the study. The study was approved by the French ethical committee, so-called *Comité de Protection des Personnes (CPP) Sud-Est 6* – France [AU 1154; 4 May 2015]. All authors had access to the study data and reviewed and approved the final manuscript.

Design of the study

In this prospective multicenter study, all CD patients requiring ileocolonoscopy, regardless of the indication, were consecutively included between September 2015 and September 2016. Besides patients' characteristics, clinical and endoscopic data were gathered. Segmental ileal and total Crohn's disease endoscopic index of severity (CDEIS)[17] were calculated for all patients. Patients with at least one ulcer in the ileum were declared to have ileal endoscopic activity. In addition, samples from blood and saliva were collected the day of colonoscopy. Stools collection was performed before bowel cleansing for colonoscopy. The procedure for taking biopsies was standardized. All the patients had ileal biopsies from macroscopically normal areas (no endoscopic lesion as defined by the GETAID consensus[18]) and additional biopsies from the edge of ulceration in case of endoscopic activity. For each patient, biopsies and stools were systematically placed in a dry tube for CEACAM6 quantification and, in MEM supplemented with 15% glycerol for microbiological analysis.

Microbiological analyses

Number determination of total *E. coli* associated with ileal mucosa and in stool

Two ileal biopsies were taken from the edge of ulceration in case of endoscopic lesions or randomly within the ileum in case of no lesion. Ileal biopsies were washed in phosphate-buffered saline (PBS), crushed (Ultra-Turrax, IKA) and incubated for 15 min on a tube rotator at room temperature in the presence of Triton 0.1X. Ten-fold dilutions of the lysate were then plated on Drigalski agar to number total *E. coli* colonies after 24 h of incubation at 37°C. Results are given in colony forming unit (cfu)/ileal biopsy. The validation of *E. coli* identification was carried out by mass spectrometry.

The stools samples, stored at −80°C in 15% glycerol Minimum Essential Medium (MEM), were crushed in physiological water. Ten-fold dilutions of the lysate were then plated on Drigalski agar to number total *E. coli* colonies after 24 h of incubation at 37°C. Results are given in colony forming unit (cfu)/mg. A random selection of 45 lactose positive bacteria was perform on Drigalski plate and *E. coli* identification was confirmed by mass spectrometry using "Vitek MS System" (Biomerieux).

Pre-screening invasion test

A pre-screening test was realized using 45 *E. coli* strains isolated per sample (ileal biopsies or stools). These strains were mixed to analyze their global abilities to invade intestine-407 epithelial cells (American Type Culture Collection, ATCC) maintained in the culture medium recommended by ATCC. Briefly, a maximum of 45 strains per sample were equitably and extemporaneously mixed to infect I-407 cells at a multiplicity of infection equal to 100 bacteria per cell (MOI100) during a 3-h period, following by 1 h of gentamicin exposure (100 μg/ml) to kill extracellular bacteria. Cells were then lysed using triton 1X and the number of internalized *E. coli* of each mixture was determined on Drigalski agar plate. K-12 (non-AIEC *E. coli* strain) was use as negative control, and LF82 (reference strain of AIEC) was used as positive control. Results are given in colony forming unit (cfu)/mL.

Characterization of AIEC bacteria

Phenotypical assays to identify AIEC bacteria

The AIEC characterization (i.e., the definition of AIEC-positive patients) has been carried out by analyzing their abilities to adhere to and to invade intestinal epithelial cell lines, as well as to survive and replicate within macrophage cell lines, by conducting gentamicin

protection assays with intestine-407 epithelial cells (ATCC, CCL-6) and THP-1 macrophages (ATCC, TIB-202), as previously described.[6,19] For all phenotypical assays, the non-AIEC *E. coli* strain, K-12, was use as negative control, and the AIEC reference strain LF82 was used as positive control.

Enterobacterial repetitive intergenic consensus polymerase chain reactions (ERIC-PCR)

ERIC-PCR were performed for CD-associated AIEC as previously described[20,21] to assess the number of AIEC clonal strains. Briefly, 2 ml of bacterial culture was centrifuged at 2800 g for 5 min and the bacterial pellet has been suspended in 200 µL of distilled water and lysis at 95°C during 10 min. Cellular waste has been removed after centrifugation at 21,000 g for 5 min. Bacterial lysate was used for ERIC-PCR with a reaction mixture containing AAG TAA GTG ACT GGG GTG ACG G primer at a concentration of 10 µmol/L, FirePol MasterMix 5X®, and distilled water. The PCR program used was: First cycle: Denaturation at 94°C for 5 min/First time to set primers at 36°C for 1 min/Next 36 cycles: Elongation at 72°C for 3 min/ Denaturation at 92°C for 1 min/Fixed primers at 36°C for 1.5 min/ Final elongation: at 72°C for 10 min. PCR products were revealed following migration on a 1% agarose gel.

Anti-total *E. coli* antibodies measurements

Anti-total *E. coli* antibodies (AEcAb) were measured in the serum using indirect ELISA test on whole bacteria. Serum were tested against *E. coli* MG1655 strain. Briefly, 1.6×10^7 bacteria were distributed on an ELISA plate (100 µL/well) and then allowed to dry overnight at 37°C for a "dry coating." The wells were emptied by inversion, washed twice in washing buffer (PBS-Tween 20 0.05%), saturated with blocking buffer (PBS-milk 5%, 100 µL/well) for 2 h at room temperature. The wells were then washed once in washing buffer and 50 µL/well of sample (serum serial dilutions in blocking buffer, 1/10 to 1/95,367.4) were incubated for two hours at room temperature. The wells were then washed three times in washing buffer and incubated two hours at room temperature with HRP-conjugated goat anti-human IgG secondary antibody (Sigma-Aldrich; 1/50,000, 50 µL/well). The wells were washed three times in washing buffer and incubated 30 min at room temperature, protected from light, with the substrate (TMB, H_2O_2; in sodium acetate buffer, 13.6 g/L, pH 6). Finally, 50 µL/well of H_2SO_4 1M were added and the absorbance at 450 and 570 nm were measured using a spectrophotometer. The results obtained for each serum dilution range were plotted on a graph [Delta absorbance (450–570 nm) = *f* (dilution log)] with the GraphPad software (GraphPad Inc.). A variable slope (4-parameters) curve was fitted to experimental values to determine the sample antibody titer defined as serum concentration at the inflexion point (effective concentration 50, EC50). Human serum AB (Sigma-Aldrich, H4522) was included on each plate as a control. The data obtained for each patient were normalized with respect to the AB serum.

Quantification of CEACAM6 from ileal biopsies and saliva

After a careful mouth rinsing with water, CD patients salivated in a dedicated jar (at least 1 ml of saliva). The salivary sample was quickly centrifuged at 1000 g during 5 min at 4°C. The supernatants were then collected and stored at −80°C.

Ileal biopsies in healthy and ulcerated areas were taken and extemporaneously dry frozen in liquid nitrogen and then stored at −80°C. For CEACAM6 measurement, ileal biopsies were washed in PBS and crushed (Ultra-Turrax, IKA) on ice in the presence of Triton 1%. The homogenates were then centrifuged at 3000 g during 5 min at 4°C. The supernatants were then collected and stored at −80°C. Protein concentrations were determined by Bradford assay.

The levels of CEACAM6 from ileal biopsies and saliva were measured (duplicates) using Human CEACAM-6/CD66c DuoSet ELISA (R&D), blinded from the clinical, biological and endoscopic data. Results were given in pg/mg of total proteins.

Data management and statistical analysis

Study data were collected and managed using Research Electronic Data Capture (REDCap) at Clermont-Ferrand University Hospital. REDCap (Research Electronic Data Capture) is a secure, web-based application designed for data capture for research studies, providing (1) intuitive interface for validated data entry; (2) audit trails for tracking data manipulation and export procedures; (3) automated export procedures packages; and (4) procedures for importing data from external sources.

Statistical analysis was performed using Stata software (version 13, StataCorp). The tests were two-sided, with a Type I error set 5%. Baseline features were presented as mean ± standard-deviation or median [interquartile range] according to statistical distribution. The assumption of normality was assessed by Shapiro-Wilk test. Comparisons of patients being characterized by the independent groups were performed using the chi-squared or Fisher's exact tests for categorical variables and using the Student *t*-test or the Mann-Whitney test for quantitative parameters (homoscedasticity verified with Fisher-Snedecor test). Multivariable analysis was carried out to identify factors associated to AIEC + patients. The correlations between quantitative parameters were investigated using Spearman test according to statistical distribution. The Sidak's type I error correction was applied to take into account multiple comparisons. The level of correlation was expressed by correlation coefficient (ρ) with *p*-values. Receiving operator curves (ROC) were used to define the best cut-off values to detect the AIEC-positive patients. Sensitivity, specificity, positive

predictive values (PPV) and negative predictive values (NPV) were given with 95% confidence interval [95% CI]. Positive and negative likelihood ratios were also mentioned.

RESULTS

Baseline characteristics of the population

A total of 102 CD patients were enrolled in eight centers (see Appendix 1). Their baseline characteristics are given in Table 1. Among them, 36.3% (37/102) had active lesions in the ileum while 53.8% (55/102) had endoscopic activity defined as at least one ulceration.[18] The mean segmental ileal and total CDEIS were 4.8 ± 7.1 and 2.6 ± 3.9, respectively.

Prevalence of ileal AIEC colonization and its associated factors

Overall, AIEC bacteria colonized the ileum of 24.5% (25/102) of patients with CD. We did not observe any clinical factor associated with AIEC infection among baseline characteristics (Table 1). Except for a numerically relevant but not statistically significant decreased prevalence in patients treated with anti-TNF (18.8% vs. 31.2%, $p = 0.21$), there was no relationship between current medication and colonization by AIEC (Table 1). There was also no link between AIEC colonization and clinical activity (mean CDAI: 110 ± 99 vs. 144 ± 121, $p = 0.29$), fecal calprotectin level (194.1 ± 180.4 vs. 254.8 ± 367.2, $p = 0.53$), segmental ileal CDEIS (5.1 ± 6.8 vs. 4.7 ± 7.3, $p = 0.82$) or total CDEIS (2.3 ± 2.6 vs. 2.7 ± 4.4, $p = 0.60$). AIEC was present at least in 29.7% (11/37) of patients with active ileal lesions and in 21.5% (14/65) of patients with no ileal lesions ($p = 0.35$). In multivariable analysis, we did not identify any factor associated with AIEC infection.

Detection and characterization of AIEC strains within ileal biopsies of patients with CD

The ERIC-PCR profiles showed that 37.5%, 40.6%, and 29.5% of the AIEC-positive patients were colonized by one, two or three clonal AIEC strains, respectively (Figure 1).

Abundance and global invasive ability of total *E. coli*

To overcome the time-consuming process of AIEC characterization from ileal biopsies, we searched for faster procedures. Then, we assessed the abundance (enumeration of total *E. coli*) and global invasive ability of *E. coli* ileal specimens of CD patients.

The number of total *E. coli* associated to the ileum was increased in CD patients with prior intestinal resection ($p = 0.03$). There was no other clinical factor associated to this number. The abundance (Figure 2a) of ileal total *E. coli* was significantly higher in AIEC-positive patients, comparatively to AIEC-negative patients ($p = 0.0065$). Using a ROC curve (area under the curve (AUROC) = 0.70 [0.61–0.77]), we determined the best threshold of total *E. coli* in the ileum to detect the presence of ileal AIEC bacteria (Supplementary Figure S1). The cut-off value of 60 cfu/biopsy demonstrated the best performances to detect the presence of ileal AIEC bacteria with high sensitivity (91.7% [7.3–99.0]) and negative predictive value (94.1% [80.3–99.3]) (Table 2). It could be a reliable test for AIEC screening, avoiding to perform time-consuming procedures in 39.5% of the patients (=proportion of patients with cut-off value < 60 cfu/biopsy).

While the gold standard is to evaluate the invasive property of each isolated strain, we assessed the global invasive ability (median invasive ability of a mix of 45 strains) as alternative process (Figure 2b). The global invasive ability of ileal total *E. coli* was significantly higher in AIEC-positive compared to AIEC-negative patients ($p = 0.0007$). Using a ROC curve, we determine that a threshold above 9000 internalized bacteria was able to detect the presence of AIEC with the following specificity (91.7% [72.8–98.7]) and positive predictive value (80.0% [55.2–100.0]) (Table 2). The sequential use of the two assays demonstrated negative and positive predictive values of 88.5% [80.5–96.5] and 80.0% [55.2–100.0], respectively (Table 2).

Ileal level of CEACAM6

We hypothesized that overexpression of CEACAM6 in the ileum could be a potential biomarker to select CD patients colonized by ileal AIEC. While we observed a correlation between the number of total *E. coli* associated to the ileum and the level of ileal CEACAM6 ($\rho = 0.25$, $p = 0.028$) (Figure 3a), we did not find any difference regarding the level of ileal CEACAM6 between AIEC-positive and AIEC-negative patients ($p = 0.96$) (Figure 3b) or between biopsies taken from healthy or ulcerated zone ($p = 0.24$) (Supplementary Figure S2). In multivariable analysis, we did not identify any factor related to patients' characteristics, current medication or disease activity that was associated with the ileal level of CEACAM6. Among the 25 AIEC-positive patients, ileal level of CEACAM6 was positively correlated with the abundance of ileal total *E. coli* ($\rho = 0.4000$, $p = 0.036$) (Figure 3c), while this correlation was not observed in AIEC-negative patients ($\rho = 0.0076$, $p = 0.48$) (Figure 3d).

Non-invasive tools to detect AIEC-positive patients

Blood samples

Serum anti-total *E. coli* antibodies (AEcAb) titers were measured using an indirect ELISA test on whole bacteria. The median level of

TABLE 1 Baseline characteristics of the 102 CD patients included in the study

	All patients (*N* = 102)	AIEC-positive (*n* = 25)	AIEC-negative (*n* = 77)	*p*-value
Female gender *n*, %	56 (56.6%)	48.0%	52.0%	0.24
Active smokers	34 (33.3%)	40.0%	30.8%	0.33
Montreal classification				
Disease location				
L1	29 (28.4%)	28.0%	26.8%	0.86
L2	11 (10.8%)	8.0%	11.9%	-
L3	61 (59.8%)	64.0%	61.9%	-
L4	7 (6.9%)	-	-	-
Disease behavior				
B1	55 (54.5%)	54.2%	54.7%	0.86
B2	29 (28.7%)	25.0%	25.6%	
B3	18 (17.8%)	20.8%	18.8%	
Perianal lesions	21 (20.6%)	12.0%	26.5%	0.14
Prior intestinal resection	45 (44.1%)	48.0%	41.2%	0.56
Current therapies				
5-ASA	7 (6.9%)	8.0%	5.9%	0.71
Corticosteroids	12 (11.8%)	12.0%	11.7%	0.67
Immunosuppressive therapies	34 (33.3%)	20.0%	25.0%	0.61
Anti-TNF agents	35 (34.3%)	18.8%	31.2%	0.21
Other biologics	3 (2.9%)	4.0%	2.9%	0.96
CDAI, mean ± SD	131 ± 109	110 ± 99	144 ± 121	0.29
CRP, g/L mean ± SD	8.1 ± 13.8	7.4 ± 11.2	8.1 ± 14.1	0.98
Fecal calprotectin, µg/g mean ± SD	244 ± 358	194.1 ± 180.4	254.8 ± 367.2	0.53

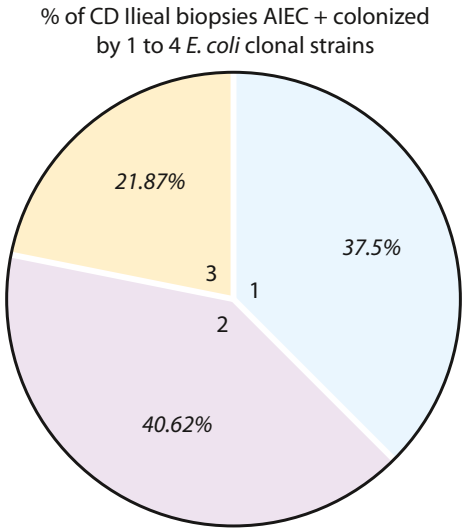

FIGURE 1 Clonality of adherent and invasive *E. coli* strains isolated from Crohn's disease patients as assessed by Enterobacterial repetitive intergenic consensus polymerase chain reactions

AEcAb was two-fold higher in AIEC-positive patients compared to AIEC-negative patients (5.58×10^{-3} vs. 2.60×10^{-3}; $p = 0.038$) (Figure 4). Using a ROC curve (Supplementary Figure S3), we determine that a cut-off value > 1.9×10^{-3}, demonstrated a very high sensitivity (94.7% [73.2–100.0]) and negative predictive value (96.6%[89.9–100.0]) (Table 3). This test could avoid to perform time-consuming assays in 33.3% of the patients (rate of true negative). In contrast, we did not observe any correlation between the level of AEcAb and the number of ileal total *E. coli* ($\rho = -0.07$, $p = 0.56$) or the level of ileal CEACAM6 ($p = 0.01$, $p = 0.93$) (data not shown).

Stools samples

We did not observe any difference of fecal abundance of total *E. coli* in AIEC-positive and AIEC-negative patients (Figure 5a). In the same way, fecal *E. coli* strains were not more invasive (Figure 5b) in AIEC-positive compared to AIEC-negative patients. These results suggest

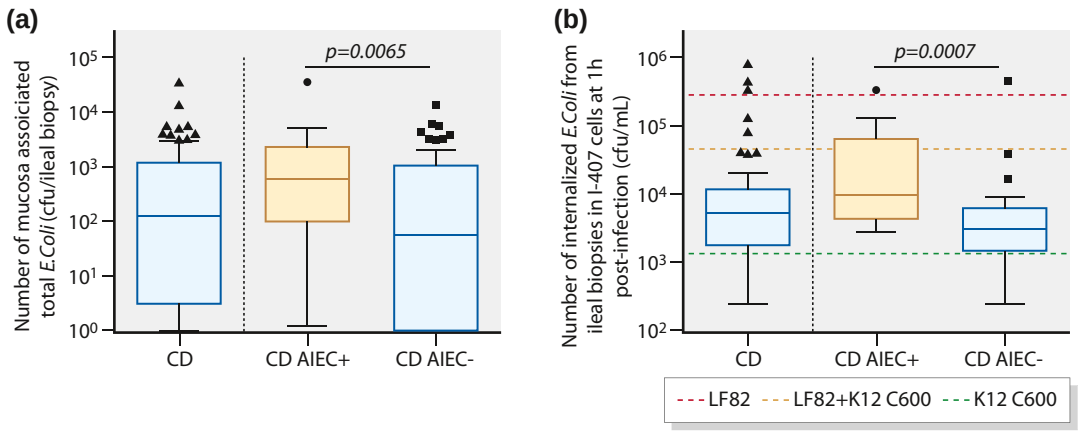

FIGURE 2 Abundance (a) and global invasive ability (b) of ileal *E. coli* strains isolated from 102 Crohn's disease patients included in the study. Abundance of total mucosa associated *E. coli* are determined in ileal biopsies for each patient and given in colony forming unit (cfu)/biopsy (a). A pre-screening test was realized on 45 *E. coli* strains mixture per ileal biopsy for each patient to analyze their abilities to invade intestine-407 epithelial cells (b). For pre-screening test, the number of internalized *E. coli* was determined after 3 h of infection plus 1 h post-infection by a gentamycin protection assay. The non-adherent and invasive *E. coli* (AIEC) strain, K-12, was use as negative control, and the AIEC reference strain LF82 diluted or not in a non-AIEC *E. coli* strain, K-12 (1:48), was used as positive control. Statistical analysis was performed using Mann Whitney test and figures were performed using Tukey grouping

TABLE 2 Performances of ileal-associated *E. coli* enumeration, global invasive ability and both used sequentially to detect patients colonized by adherent and invasive *E. coli* (AIEC) in the ileum

Cut-Off Value	Sensitivity	Specificity	NPV	PPV	LR+	LR−
Ileal-associated *E. coli* enumeration						
60 cfu/biopsy	91.7%	47.1%	94.1%	37.9%	1.73	0.17
95% CI	73.0–99.0	34.8–959.6	80.3–99.3	25.5–51.6	1.34–2.23	1.34–2.23
Global invasive ability						
>9000	61.5%	91.7%	81.5%	80.0%	7.38	0.42
95% CI	35.4–82.2	72.8–98.7	66.8–96.1	55.2–100.0	1.83–29.79	0.21–0.84
Sequential use of the two tests[a]						
	53.3%	96.4%	88.5%	80.0%	14.9	0.48
	30.2–75.1	87–99.6	80.5–96.5	55.2–100.0	3.53–63.08	0.28–0.83

Abbreviations: CI, confidence interval; LR+, positive likelihood ratio; LR−, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

[a]The global invasive assay was performed only in patients with more than 60 cfu/biopsy.

that identification of fecal *E. coli* cannot be used as a non-invasive biomarker to detect the presence of AIEC strains in the ileum of CD patients.

Salivary samples

The level of salivary CEACAM6 was positively correlated with the level of CEACAM6 in the ileum (Figure 6a; $\rho = 0.47$, $p < 0.0001$) in both macroscopically healthy areas ($\rho = 0.53$, $p < 0.0001$) and ulcerated zones ($\rho = 0.39$, $p = 0.0082$) (Supplementary Figure S4). As for ileal CEACAM6, the level of salivary CEACAM6 in AIEC-positive patients was not different from that of AIEC-negative patients ($p = 0.45$) (Figure 6b).

DISCUSSION

In this study, we reported a prevalence of at least 24.5% of CD patients colonized by AIEC and found that more than two thirds of AIEC-positive patients are colonized by ≤ 2 AIEC strains. We identified faster procedures for AIEC detection from ileal biopsies. We observed that stool samples were not appropriate to screen patients for AIEC colonization, while the measurement of AEcAb level could be a less invasive biomarker to screen CD patients for ileal colonization by AIEC.

The prevalence of AIEC (24.5%) in our cohort is consistent with the original paper from Darfeuille-Michaud and colleagues reporting a colonization by AIEC in 21.7% of the patients with chronic ileal lesions (from surgical specimens) and 36.4% in the

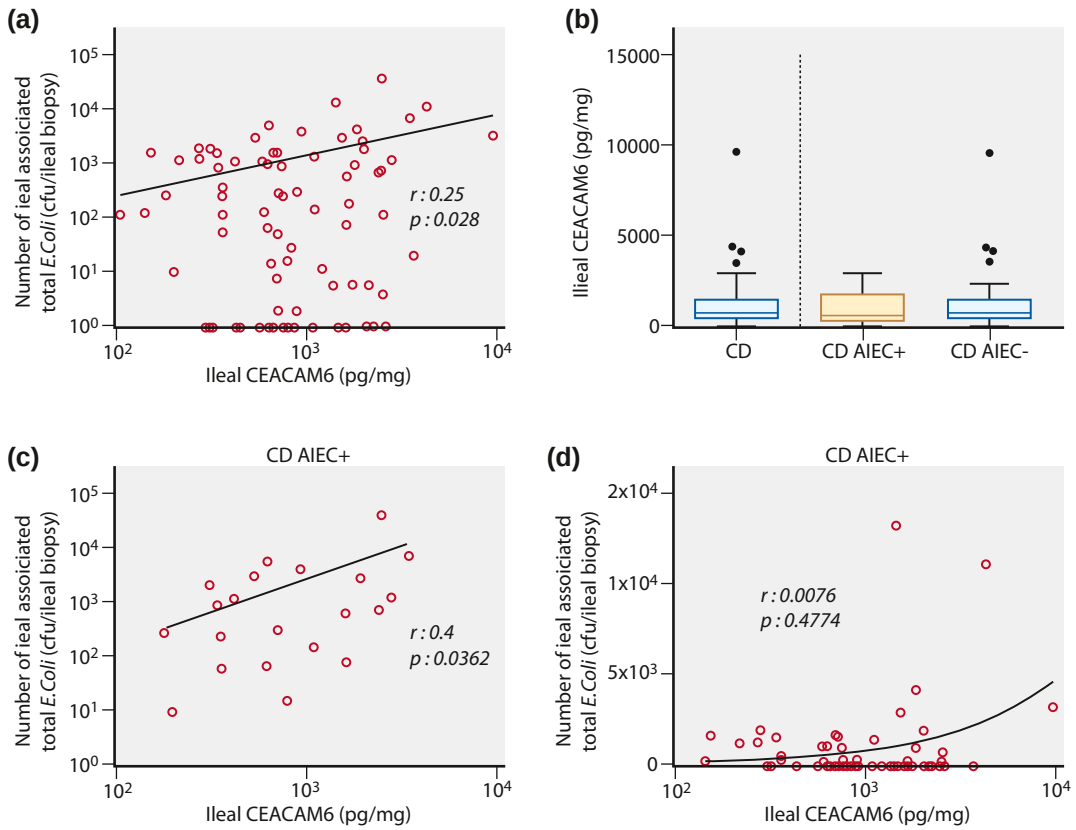

FIGURE 3 Correlation between the level of ileal Carcinoembryonic Antigen Related Cell Adhesion Molecule 6 (CEACAM6) and the number of ileal-associated total *E. coli* in the whole cohort (a), in adherent and invasive *E. coli* (AIEC)-positive (c) and AIEC-negative patients (d). For each patient, biopsies were placed in a dry tube for CEACAM6 quantification and, when possible, in MEM supplemented with 15% glycerol for microbiological analysis (n = 21 Crohn's disease AIEC-positive and n = 59 AIEC-negative). Comparison of the level of ileal CEACAM6 in AIEC-positive and AIEC-negative patients (b). Statistical analyses were performed using Mann Whitney test and figure are performed using Tukey grouping. Spearman test was used for correlation analysis

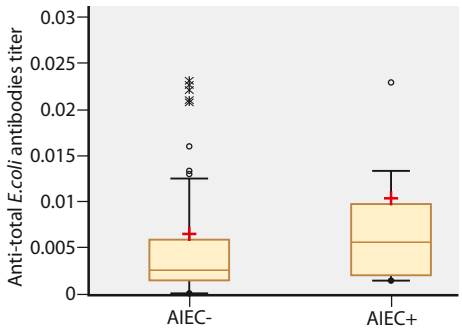

FIGURE 4 Level of anti-total *E. coli* antibodies in Crohn's disease patients with or without ileal colonization by adherent and invasive *E. coli*

neo-ileum of CD patients who had undergone ileocolectomy.[6] Since, independent cohorts from all around the world found a prevalence ranging from 21.7% to 62.5% in CD patients.[6,8–10] In a recent meta-analysis the pooled prevalence of AIEC among CD patients was 29% (95% CI 0.17–0.45).[11] We did not observe any clinical phenotype related to AIEC infection, especially no relationship between disease activity and AIEC colonization. It could suggest that AIEC colonization is not a consequence of inflammation. In the present study, we found, for the first time that AIEC colonization is often clonal as more than two thirds of the patients colonized by AIEC were infected by two or less different AIEC strains whereas none showed four or more strains. This observation could facilitate the development of AIEC-targeting drugs whose modalities could be different according to the number of targets (one AIEC strain or more).

The identification of AIEC colonization could be warranted in the management of Crohn's disease, particularly if treatments targeting AIEC, which have shown their effectiveness in various preclinical models,[22–24] confirmed its efficacy in CD patients. Indeed, several randomized clinical trials are in progress. First, the TEOREM trial (NCT02620007) is a multicenter randomized placebo-controlled trial assessing a combination of ciprofloxacin and rifaximin to achieve endoscopic response in patients with ileal CD colonized by AIEC. After a conclusive phase 1 trial, a phase 2 trial is currently assessing the effectiveness and the safety of a FimH blocker (FimH being a virulence factor of AIEC bacteria), to prevent endoscopic post-operative recurrence in CD (NCT03709628 and NCT03943446). Finally, a phase 2, double-blind, placebo-controlled trial is currently investigating the effect of an AIEC-specific bacteriophage cocktail

TABLE 3 Performances of serum anti-*E. coli* antibodies (AEcAb) to detect patients with ileal colonization by adherent and invasive *E. coli* (AIEC) in Crohn's disease

Cut-off value	Sensitivity	Specificity	NPV	PPV	LR+	LR−
1.9×10^{-3}	94.7%	43.1%	96.6%	32.7%	1.66	0.12
95% CI	73.2–100.0	31.8–55.2	89.9–100.0	20.3–45.1	1.31–2.10	0.02–0.84

Abbreviations: CI, confidence interval; LR+, positive likelihood ratio; LR−, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

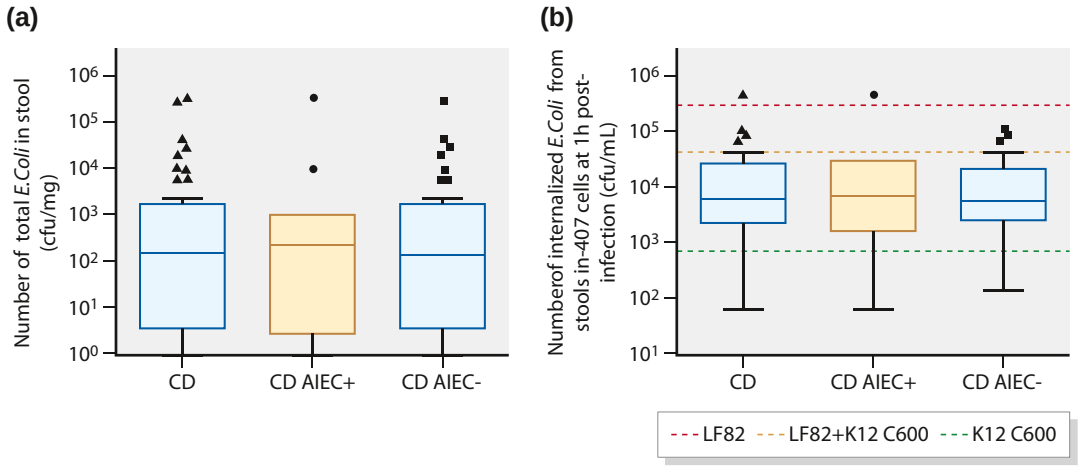

FIGURE 5 Abundance (a) and global invasive ability (b) of fecal *E. coli* strains isolated from 102 Crohn's disease patients included in the study. Abundance of total *E. coli* are determined in stools for each patient and given in colony forming unit (cfu)/mg (a). A pre-screening test was realized on 45 *E. coli* strains mixture per stool for each patient to analyze their abilities to invade intestine-407 epithelial cells (b). For pre-screening test, the number of internalized *E. coli* was determined after 3 h of infection plus 1 h post infection by a gentamycin protection assay. The non-adherent and invasive *E. coli* (AIEC) strain, K-12, was use as negative control, and the AIEC reference strain LF82 diluted or not in a non-AIEC *E. coli* strain, K-12 (1:48), was used as positive control. Statistical analysis was performed using Mann Whitney test and figures were performed using Tukey grouping

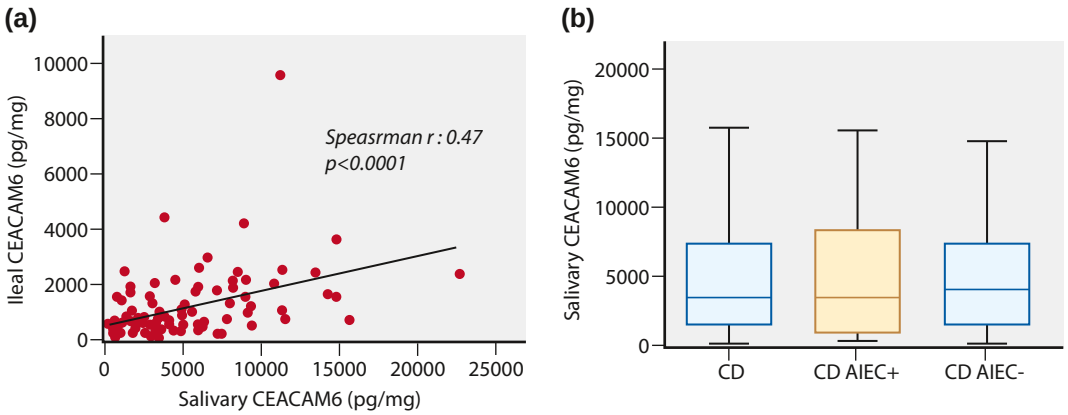

FIGURE 6 Carcinoembryonic Antigen Related Cell Adhesion Molecule 6 (CEACAM6) level in saliva of the Crohn's disease patients included in the study. For each patient, when possible, saliva supernatant and biopsies were placed in a dry tube for CEACAM6 quantification ($n = 94$ Crohn's disease [CD] patients). (a) The level of salivary CEACAM6 was determined by ELISA and was correlated with the level of ileal CEACAM6. (b) The level of ileal CEACAM6 was not different in adherent and invasive *E. coli* (AIEC)-positive and AIEC-negative patients with CD ($n = 20$ and $n = 60$, respectively)

(EcoActive) on disease activity, inflammatory markers (CRP and fecal calprotectin) and AIEC load in patients with CD (NCT03808103). The identification of AIEC + patients is a key point in this kind of strategies. A rapid and non-invasive biomarker of ileal colonization by AIEC could facilitate the recruitment of eligible patients in these trials and could favor research initiative on this topic.

The current method to screen the patients for AIEC identification is a real limitation. First, this requires to perform a colonoscopy for taking ileal biopsies. It has been reported in a nationwide study assessing the acceptability of the monitoring tools by 916 IBD patients that this procedure can be felt as a burden by the patients and that blood puncture or stool collection are more accepted.[15] The second issue is the time to obtain the results of the identification. As the definition of AIEC is strictly based on their phenotype, the search for AIEC needs several tedious and time-consuming laboratory steps such as bacteria isolation, test of adhesion, test of invasion and evaluation of survival within macrophages. The duration of this process ranges from 4 to 6 weeks depending on the presence of a full-time dedicated lab technician. Then, making the identification of AIEC-positive patients easier, remains a challenge for IBD researchers. To overcome this difficulty, according to the positive correlation between the colonization by AIEC and the abundance of ileal total *E. coli*, we showed that the number of total *E. coli* associated to ileal mucosa (cut-off value < 60 cfu/biopsy) allows to strongly predict the absence of AIEC (with high negative predictive value) and then is a reliable test for AIEC screening. These data could avoid performing such time-consuming procedures in more than one third of the patients. In addition, the global invasive ability that is the median invasive ability of a mix of 45 strains could be a faster alternative process to assess the invasive phenotype of the bacteria with nice positive predictive value (80%).

Stool collection, which is more convenient for IBD patients than colonoscopy,[15] is an intuitive candidate for non-invasive identification of AIEC-colonized CD patients. However, we did not find any correlation between ileal AIEC colonization and either the number of fecal total *E. coli* or the global invasive properties of fecal *E. coli*. All these data suggest that fecal screening for AIEC is not a suitable alternative for screening AIEC-positive patients, that is, with ileal colonization by AIEC and can definitely not replace identification of AIEC from ileal biopsies. This point should be taken into account in future studies warranting to select CD patients at risk to be colonized by AIEC and eligible for anti-AIEC therapeutic strategies.

AIEC bacteria are known to interfere with innate immunity.[25] Antibodies against *E. coli* antigens such as the flagellin and the outer membrane protein OmpC have been detected in CD patients from independent cohorts.[16,26–28] As collecting blood samples is a common and convenient procedure for IBD patients,[15] we looked for serum biomarkers of AIEC colonization. We reported a higher level of AEcAb in AIEC-positive patients, supporting that an antibody response against *E. coli* bacteria could exist in AIEC colonized patients. This was in line with several pre-clinical studies which suggested that AIEC interfere with adaptive immunity in inflammatory context using in vitro and in vivo laboratory models of CD.[25] Our detection of AEcAb is targeting a whole *E. coli* strain. Even if the bacterial antigens involved are not clearly identified yet, serum AEcAb level of CD patients could indirectly reflect their degree of AIEC mucosal colonization. Thus, this increased reactivity supports our hypothesis that anti-*E. coli* responses should be quantitatively and/or qualitatively different in AIEC-positive patients, probably due to the virulence properties of AIEC bacteria (invasiveness, ability to escape phagolysosomes and autophagic degradation) that may reinforce the host immune response. A more selective approach focusing on the identification of immunodominant antigens in AIEC bacteria will be investigated in future works. Even if further investigations are needed to confirm these results, this biomarker had a high negative predictive value to exclude AIEC colonization. This biomarker would avoid to perform a burdensome procedure of AIEC identification in one third of the patients and facilitate the selection and the recruitment of patients in clinical trials assessing therapies targeting AIEC.

The main limitation of our study is that none of the non-invasive biomarker was able to detect AIEC-positive patients with high positive predictive value. However, our study has several strengths. We provided original data with an appropriate sample size from a multicenter prospective study. It was the first and the largest data published so far, confirming that identification of fecal AIEC cannot replace identification of AIEC from ileal biopsies and identifying that most of AIEC infection are mono- or bi-clonal (≤2 strains). We also found faster and less invasive methods to identify AIEC-positive patients with a promising role of anti-*E. coli* antibodies.

In conclusion, CD-associated AIEC were detected in almost a quarter of patients in our cohort. More than two thirds of these patients were colonized by ≤ 2 AIEC strains. While stool samples are not appropriate, the level of anti-*E. coli* antibodies (AEcAb) could be an attractive biomarker to screen patients for AIEC ileal colonization by AIEC.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

GUARANTOR OF THE ARTICLE

Anthony Buisson.

AUTHOR CONTRIBUTIONS

A Buisson: study concept and design; scientific assays achievement; acquisition of data; analysis and interpretation of data; drafting of the manuscript. E. Vazeille: study concept and design; scientific assays

achievement; acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. X. Hébuterne, B. Pariente, S. Nancey, P. Seksik, L. Peyrin-Biroulet, M. Allez, J. Filippi, C. Yzet, M. Nachury, G. Boschetti: study concept and design; critical revision of the manuscript for important intellectual content. N. Ballet: study concept and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. E. Billard: scientific assays achievement; acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. A. Dubois, S. Rodriguez, C. Chevarin: scientific assays achievement; acquisition of data; analysis and interpretation of data. M. Goutte, G. Bommelaer: study concept and design; critical revision of the manuscript for important intellectual content. B. Pereira: analysis and interpretation of data; computation of statistical analyses. N. Barnich: study concept and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. All authors approved the final version of the article, including the authorship list.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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APPENDIX 1

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