Original Article

Utility of clinical and laboratory data to estimate the probability of bacterial diarrhea diagnosed by stool multiplex Polymerase Chain Reaction assay in a pediatric population

Impiego di dati clinici e di laboratorio per stimare la probabilità di diarrea ad eziologia batterica diagnosticata mediante multiplex Real-Time PCR su campioni di feci in una popolazione pediatrica

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Key words: *Campylobacter*; *Salmonella*; *Shigella*; diarrhea; real-time Polymerase Chain Reaction.

ABSTRACT

Aims: we estimated the probability of a positive result for the most common bacterial causal agents of diarrhea, such as *Campylobacter* spp., *Salmonella* spp., *Shigella*/Enteroinvasive *Escherichia coli*, *Yersinia enterocolitica* or Shiga toxin-producing *Escherichia coli* by a stool multiplex Polymerase Chain Reaction (PCR) assay in a pediatric population evaluated at the Pediatric and Pediatric Emergency Unit, Children Hospital of the Azienda Ospedaliera SS. Antonio e Biagio e Cesare Arrigo, Alessandria, Italy, during the period April 2022 - July 2023.
Methods: we analyzed the clinical data collected by the Pediatrician on the stool sample request form along with complete blood count and C-Reactive Protein (CRP).
Results: in our case series, the presence of blood/mucus in stool along with an increased value of CRP are independently associated with a positive result diagnosed by molecular method for bacterial diarrhea caused by the aforementioned pathogens.
Conclusions: the results proposed in this paper can be of help in hospital settings without the availability of a stool multiplex PCR assay to estimate the probability of bacterial diarrhea in a pediatric patient.

Obiettivi: in questo report abbiamo stimato la probabilità di un risultato positivo per i più comuni agenti causali batterici di diarrea, quali *Campylobacter* spp., *Salmonella* spp., *Shigella/Escherichia coli* enteroinvasiva, *Yersinia enterocolitica* od *Escherichia coli* produttori di tossine Shiga, identificati mediante una multiplex Real-Time PCR su campioni di feci in una popolazione pediatrica valutata presso la Struttura Complessa di Pediatria e Pronto Soccorso Pediatrico dell'Azienda Ospedaliera SS. Antonio e Biagio e Cesare Arrigo di Alessandria, nel periodo aprile 2022 - luglio 2023.
Metodi: sono stati analizzati i dati clinici inseriti dal Pediatra sul modulo di richiesta di esame molecolare su campione di feci prelevate da pazienti con diarrea a sospetta eziologia infettiva contemporaneamente all'emocromo completo ed alla proteina C reattiva.
Risultati: nella nostra casistica, la presenza di sangue/muco nelle feci associata ad un elevato valore di proteina C reattiva sono risultati indipendentemente associati ad una probabilità più elevata di risultato positivo mediante metodo molecolare per i batteri descritti sopra.
Conclusioni: i risultati di questo lavoro possono essere di aiuto per stimare la probabilità di diarrea ad eziologia batterica in un paziente pediatrico in strutture ospedaliere senza la disponibilità di un test molecolare.

INTRODUCTION

Diarrhea is the passage of loose stools at an increased frequency, is defined as acute when it lasts less than two weeks, and can be categorized into infectious or non-infectious.[1] Acute infectious diarrhea is frequently caused by viral agents, such as norovirus, rotavirus, or adenovirus,[2] it is mostly watery diarrhea, and therapy is rehydration.[3] Conversely, bacterial diarrhea is often associated with more severe clinical pictures due to invasive infections, characterized by the presence of blood/mucus defined as dysentery.[2] The most frequent causal agents of bacterial diarrhea are: *Salmonella* spp., *Campylobacter* spp., *Shigella* spp., Shiga toxin-producing *Escherichia coli* (STEC), and *Yersinia* spp.[4] In some patients suffering from bloody diarrhea, while waiting for microbiology test results, empiric antimicrobial therapy can be considered or contraindicated.[3]

The gold standard for the identification of bacterial causal agents of diarrhea is stool culture, while for viral agents, immunoassays are available.[1] Regarding the latter, diagnostic accuracy has been described as quite variable by some authors, with sensitivity ranging from less than 50% to more than 90%.[5]

Concerning the pediatric population, acute diarrhea is very common, often of viral etiology,[6] and the most common remark clinicians make about testing for bacterial agents is that the results of stool cultures are often not available until two to three days after collection.[6] To speed up the process of stool testing, syndromic panels of very high diagnostic accuracy that allow simultaneous detection of several gastrointestinal pathogens by real-time Polymerase Chain Reaction (PCR) in a very short time have been available for several years.[7,8] Nevertheless, in many hospital settings, molecular methods for stool testing are not available.

The aim of this study was to estimate the probability of a positive result for the most common bacterial causal agents of diarrhea, such as *Campylobacter* spp., *Salmonella* spp., *Shigella*/Enteroinvasive *Escherichia coli* (EIEC), *Yersinia enterocolitica* or STEC by a stool multiplex PCR assay in a pediatric population. Moreover, we reported the local Minimum Inhibitory Concentration (MIC) distributions of *Campylobacter jejuni* and *Salmonella enterica* ssp. *enterica* strains isolated during the period April 2022 - July 2023 at the Microbiology Laboratory of the Azienda Ospedaliera SS. Antonio e Biagio e Cesare Arrigo, Alessandria, Italy, to help pediatricians set up a possible empirical antimicrobial therapy.

MATERIALS AND METHODS

Design of the study

This was a cross-sectional study that evaluated the period April 2022 - July 2023. Inclusion criteria: all patients evaluated at the Pediatric and Pediatric Emergency Unit, Children's Hospital of the Azienda Ospedaliera SS. Antonio e Biagio e Cesare Arrigo, Alessandria, for diarrhea of suspected infectious origin, whose stool samples were collected for testing by the multiplex PCR-based assay available at our Microbiology Laboratory. Along with the molecular test results, the main clinical data reported by the pediatrician on the sample request form and principal inflammatory parameters such as white blood cells, neutrophils, lymphocytes, and C-Reactive Protein (CRP) were evaluated.

Multiplex Polymerase Chain Reaction assay

The multiplex PCR-based assay available at our laboratory is the BioFire® FilmArray® Gastrointestinal panel (bioMérieux, Marcy-l'Étoile France), a multiplexed PCR test that targets 22 pathogens (13 bacterial, 5 viral, and 4 parasitic) in around an hour.[9] All stool samples were collected by the FecalSwab@ system (Copan Italia, Brescia, Italy) and processed according to the manufacturer's instructions as described in a previous study conducted in our institution.[10] We divided the pathogens identified by the molecular method into two groups. Group 1: *Campylobacter* spp., *Salmonella* spp., *Shigella*/Enteroinvasive *Escherichia coli* (EIEC), *Yersinia enterocolitica* and STEC; group 2: Norovirus GI/GII, Adenovirus F40/41, Rotavirus A, Sapovirus, Astrovirus, *Clostridium difficile* Toxin A/B, Enteropathogenic *Escherichia coli* (EPEC), Enteroaggregative *Escherichia coli* (EAEC), Enterotoxigenic *Escherichia coli* (ETEC) and negative result. This classification was driven by the different patient management and therapeutic approaches that can be considered or even contraindicated in relation to the pathogens included in group 1 compared to those included in group 2.[3]

Stool culture

Stool cultures were performed when requested by the Pediatrician along with the multiplex PCR-based assay or in case of its positive result for culturable pathogens. All stool samples were plated to MacConkey agar, Salmonella-Shigella agar (SS), and Campylobacter CVA agar (CVA). Only when the PCR-based assay was positive for *Yersinia enterocolitica* or STEC or that specific culture was requested by the Pediatrician, the samples were plated to Cefsulodin-Irgasan-Novobiocin agar (CIN) or MacConkey-sorbitol agar.

Identification and antimicrobial susceptibility testing

Identification of isolates was performed by Vitek 2® system (bioMérieux) or by matrix-assisted laser desorption ionization–time of flight mass spectrometry Vitek® MS (bioMérieux). Antimicrobial Susceptibility Testing (AST) of *Campylobacter* spp. was performed by a MIC method, and AST of *Salmonella* spp. and *Shigella* spp. by Vitek 2® system. EUCAST versions 12.0[11], 13.0[12], and 13.1[13] were used for the interpretation of MIC values.

Data extraction

The data were extracted by a new laboratory information system provided with features suited for the specific requirements of a Microbiology Laboratory (Concerto: Dedalus Healthcare Systems Group SpA, Firenze, Italy), that allows not only the management of the clinical specimens, but also the production of highly configurable epidemiological reports.

Statistical methods

Continuous variables were expressed as medians and Interquartile Range (IQR). Categorical variables were expressed as absolute numbers and percentages. The MIC values were displayed on the X-axis of the bar charts and expressed in mg/l. On the Y-axis, the number of isolates was reported. A comparison of median values was performed by the Mann-Whitney U test. Fisher's exact test and McNemar's test, as appropriate, were used for testing relationships on categorical variables. A Receiver Operator Characteristic (ROC) curve analysis was performed to look for possible thresholds of classifiers resulting in significant differences by comparison of median values, and Youden's J statis-

tic was applied to establish the optimal cut-off value. A binary logistic regression analysis was performed to assess possible independent predictors of the result of multiplex PCR-based assay for group 1 compared to group 2. Microsoft Excel 2010 (Microsoft Corporation, Redmond, USA) and IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, New York) were used for the analysis. The significance level was set at $p \leq 0.05$.

RESULTS

Demographic and clinical data

A total of 124 patients were included in the study. Median age was five years (IQR: 1-9), and 78/124 (62.9%) were males. The data collected at admission to the hospital are described in Table 1.

Multiplex PCR-based assay

The 61.3% (76/124) of samples were positive by Multiplex PCR-based assay. The pathogens identified are reported in Figure 1. More than half of the samples (44/76; 57.9%) were positive for *Campylobacter* spp. or *Salmonella* spp.

Stool culture

Among the 124 samples, culture was performed in 73/124 (58.9%). Of these 73, 37 (50.7%) were positive. The pathogens isolated are described in Figure 2. Figures 3 and 4 report the MIC distributions of *Campylobacter jejuni* and *Salmonella enterica* ssp. *enterica* for the antimicrobials commonly tested for these pathogens.

Comparison between molecular method and culture

The comparison between the two methods is reported in Table 2. No false negative results by multiplex PCR-based assay were observed. Among the 76 positive results by multiplex PCR-based assay, 48 (63.2%) were positive for group 1 pathogens, cultured at our laboratory. Therefore, the comparison of proportions was performed on this subgroup. Stool culture results: 37/48 (77,1%) positive *vs* 11/48 (22,9%) negative. Conversely, among the 20 samples negative by multiplex PCR-based assay, 20/20 (100%) were culture-negative (McNemar's test; $p<0.001$).

Bivariate analysis between demographic, clinical and laboratory data with multiplex PCR-based assay results

The association between demographic/clinical/laboratory data and a positive result by molecular method for group 1 pathogens (N=48) compared to group 2 (N=76) is shown in Table 3.

Table 1. Available data at presentation (N=124).

Clinical data	N	%
Discharge >3/day	111	89.5
Blood/mucus in stool	56	45.2
Nausea, vomiting or fever	91	73.4
Returned traveler	5	4
Suspect inflammatory bowel disease	24	19.4

Receiver operating characteristic analysis

The Receiver Operating Characteristics (ROC) curve of CRP as a classifier of a positive result for group 1 by molecular method is displayed in Figure 5. The best cut-off has been found at a value of 2.675 mg/dL, consistent with 70.8% sensitivity and 64.5% specificity. Youden's J statistic: 0.353.

Binary logistic regression

The results of binary logistic regression to evaluate the performance as independent predictors of positive results by molecular method for group 1 compared to group 2 of the variables significantly associated with the bivariate analysis are shown in Table 4. The model reported had a χ^2 value of 56.77 ($p<0.0001$), a Nagelkerke R Square of 0.499, and correctly predicted 79% of the overall results. According to our data series, the regression equations estimating the probability of having a positive result for group 1 pathogens by molecular method for a pediatric patient suffering from diarrhea, with (Y_1) or without (Y_2) blood/mucus in stool, with no suspect of Inflammatory Bowel Disease (IBD) in relation to the value of CRP (X) are therefore shown in Figure 6.

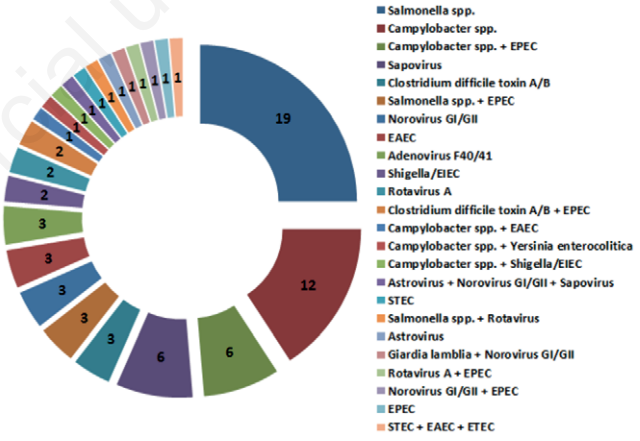


Figure 1. Pathogens identified by multiplex PCR-assay (N=76/124; 61.3%).

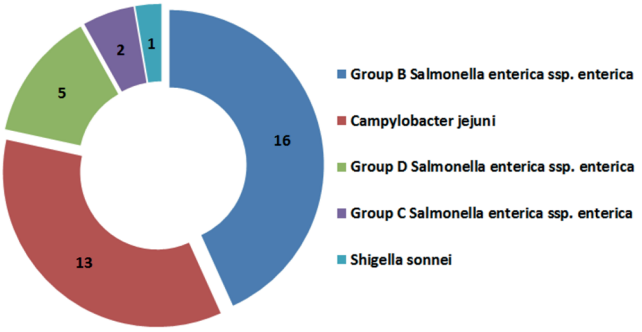


Figure 2. Pathogens isolated from stool cultures (N=37).

Table 2. Comparison of multiplex Polymerase Chain Reaction (PCR)-assay and culture results.

		Stool culture			
		Positive	Negative	Not requested	Total
Multiplex PCR-assay	Positive	37 (48.7)	16 (21.1)	23 (30.2)	76 (100)
	Negative	0 (0)	20 (41.7)	28 (58.3)	48 (100)
	Total	37 (29.8)	36 (29)	51 (41.2)	124 (100)

Data expressed as absolute numbers (row percentage).

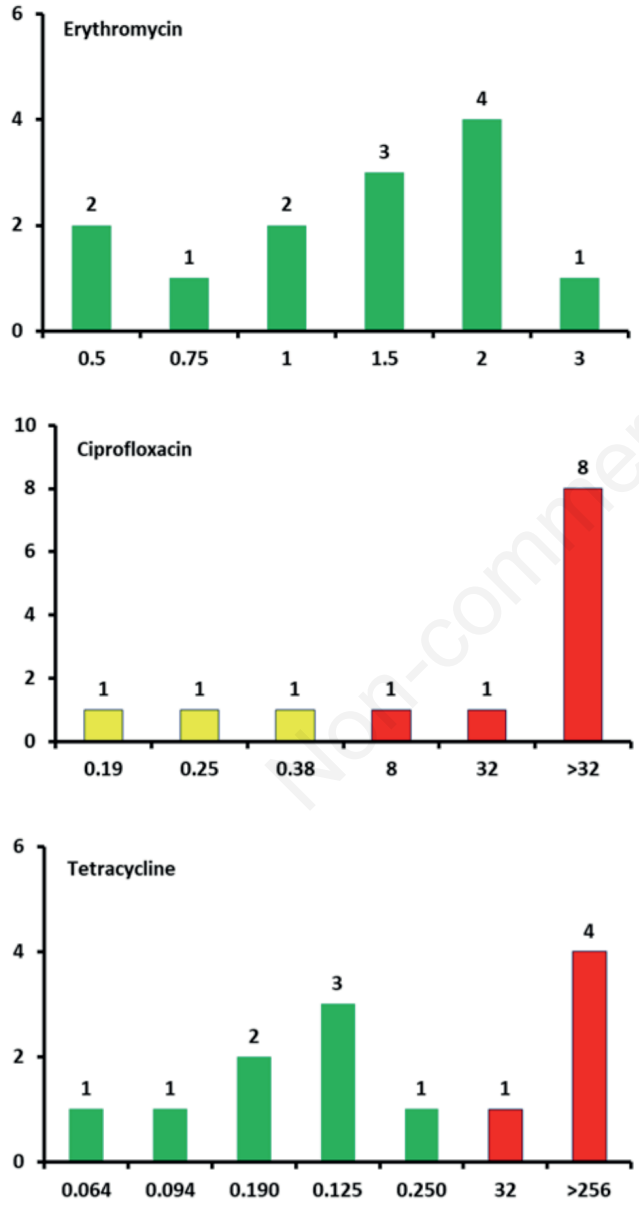


Figure 3. Minimum Inhibitory Concentration (MIC) distributions of drugs commonly tested against *Campylobacter jejuni*. Green, susceptible, standard dosing regimen; yellow, susceptible, increased exposure; red, resistant.

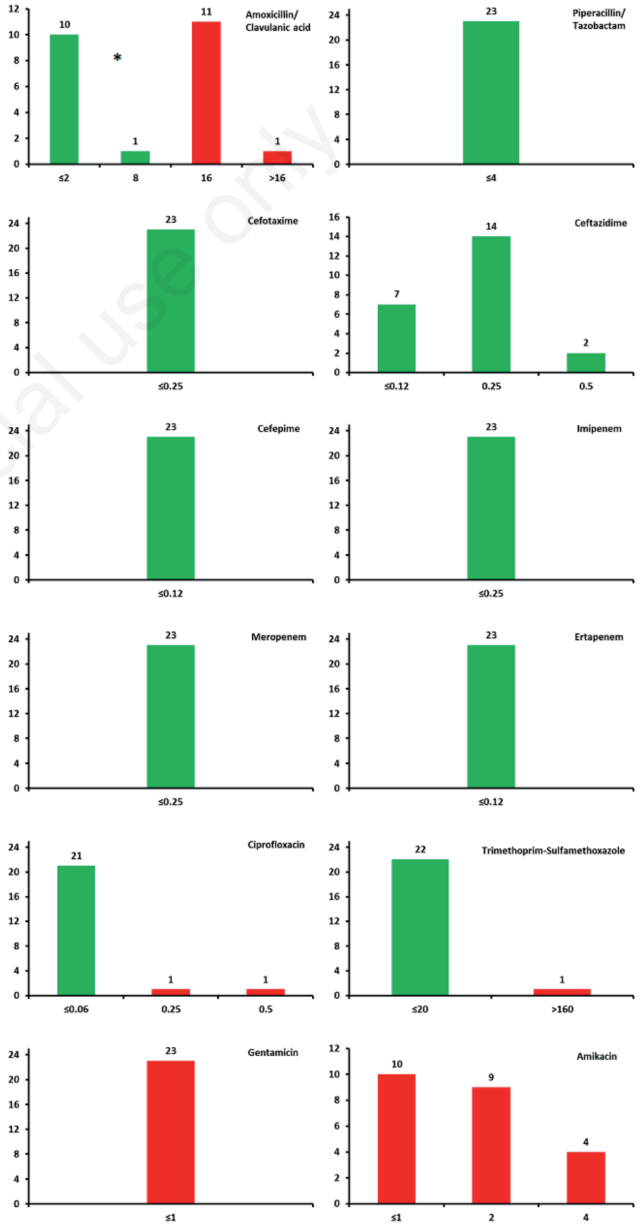


Figure 4. Minimum inhibitory concentration distributions of drugs commonly tested against *Salmonella enterica* ssp. *enterica*. Green, susceptible, standard dosing regimen; yellow, susceptible, increased exposure; red, resistant. *From EUCAST version 13.0 on, susceptible, standard dosing regimen only for intravenous therapy.

Table 3. Bivariate analysis between demographic/clinical/laboratory data and a positive result by molecular method for: *Campylobacter* spp., *Salmonella* spp., *Shigella* spp./EIEC, *Yersinia enterocolitica* or STEC (group 1) compared to other pathogens identified or negative results (group 2).

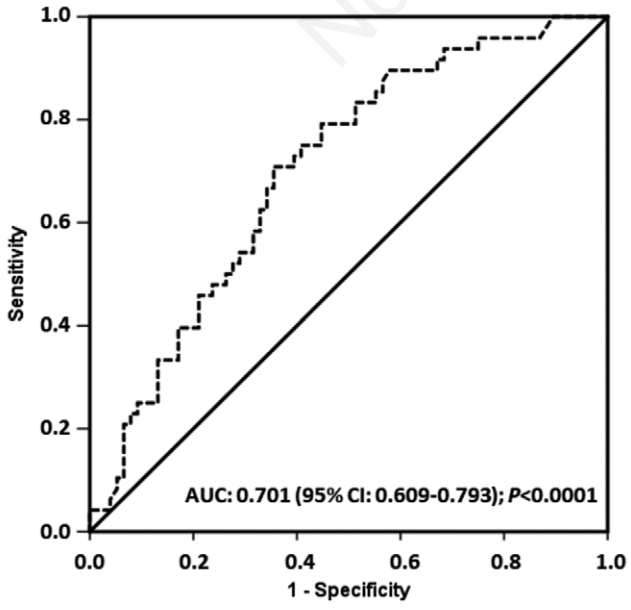
Data	Multiplex PCR-based assay		
	Group 1 (N=48)	Group 2 (N=76)	*p*
Discharge >3/day	47 (97.9)	64 (84.2)	0.016
Blood/mucus in stool	35 (72.9)	21 (27.6)	<0.0001
Nausea, vomiting or fever	41 (85.4)	50 (65.8)	0.021
Returned traveler	3 (6.3)	2 (2.6)	0.374
Suspect inflammatory bowel disease	3 (6.3)	21 (27.6)	0.004
Males	35 (72.9)	43 (56.6)	0.086
Age (years)	5.5 (2-9)	4.5 (1-10)	0.973
White blood cells (cells*10^3/mm^3)	9.2 (8.0-11.5)	10.3 (6.7-14.9)	0.585
Neutrophils (cells*10^3/mm^3)	6.0 (4.9-7.3)	6.3 (3.1-10.3)	0.908
Lymphocytes (cells*10^3/mm^3)	2.0 (1.2-3.2)	2.2 (1.3-3.7)	0.422
Neutrophil/lymphocyte ratio	2.9 (2.2-5.9)	2.7 (1.4-5.2)	0.460
C-reactive protein (mg/dL)	5.3 (1.7-11.8)	1.3 (0.2-6.3)	<0.0001

Categorical variables are expressed as absolute numbers (column percentage). Continuous variables are expressed as median (interquartile range). EIEC, *Shigella*/Enteroinvasive *Escherichia coli*; STEC, Shiga toxin-producing *Escherichia coli*.

Table 4. Binary logistic regression analysis to evaluate the performance of the variables significantly associated with the bivariate analysis as independent predictors of positive results by molecular method for *Campylobacter* spp., *Salmonella* spp., *Shigella* spp./EIEC, *Yersinia enterocolitica* or STEC (group 1 pathogens).

	β	SE	OR	(95% CI)	*p*
Discharge >3/day	1.72	1.11	5.59	(0.63-49.7)	0.123
Blood/mucus in stool	2.68	0.55	14.65	(5.01-42.83)	<0.0001
Nausea, vomiting or fever	0.98	0.62	2.68	(0.79-9.03)	0.110
Suspect inflammatory bowel disease	-1.55	0.77	0.21	(0.05-0.96)	0.044
C-reactive protein (mg/dL)	0.13	0.05	1.14	(1.04-1.24)	0.006
Constant	-4.61	1.27	0.01	.	<0.0001

EIEC, *Shigella*/Enteroinvasive *Escherichia coli*; STEC, Shiga toxin-producing *Escherichia coli*; SE, standard error; OR, odds ratio; CI, confidence interval.



Regression models

Subsequently, some examples. According to our prediction model, the probability of a positive result for group 1 pathogens by molecular method in a pediatric patient suffering from diarrhea, with blood/mucus in stool (Y_1), with a CRP value of 15 mg/dl (X) for which an IBD is not suspected, is estimated in Model #1 (Figure 7).

On the other hand, in a pediatric patient suffering from diarrhea, with blood/mucus in stool (Y_1), but with a CRP value of 0 mg/dl (X) for which an IBD is not suspected, the probability of

Figure 5. Receiver Operator Characteristic curve analysis of C-reactive Protein as classifier of a positive result by molecular method for: *Campylobacter* spp., *Salmonella* spp., *Shigella* spp./EIEC, *Yersinia enterocolitica* or Shiga toxin-producing *Escherichia coli* (group 1; N=48) compared to other pathogens identified or negative results (group 2; N=76). AUC, area under the curve; CI, confidence interval.

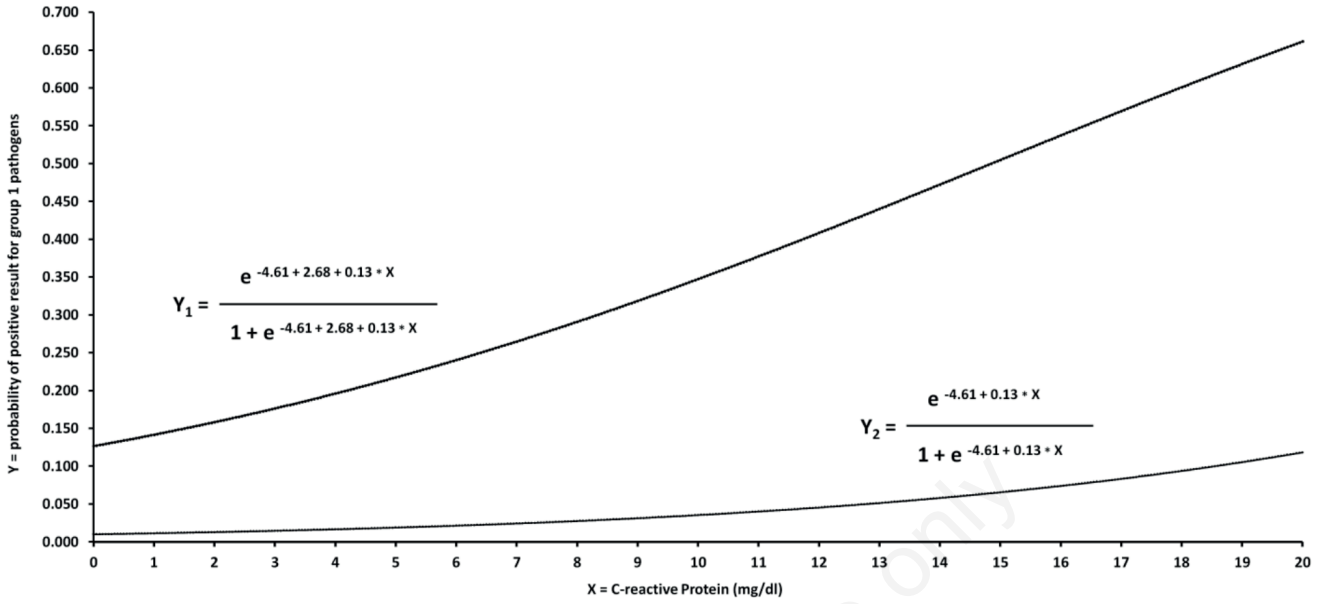


Figure 6. According to our data series, the regression equations estimating the probability of having a positive result for group 1 pathogens by molecular method for a pediatric patient suffering from diarrhea, with (Y_1) or without (Y_2) blood/mucus in stool, with no suspect of Inflammatory Bowel Disease (IBD) in relation to the value of C-Reactive Protein (CRP) (X).

having a positive result for group 1 pathogens by molecular method is estimated in Model #2 (Figure 8).

Finally, in Model #3 (Figure 9) is estimated the probability of having a positive result for group 1 pathogens by molecular method in a pediatric patient suffering from diarrhea, but without blood/mucus in stool (Y_2) and with a CRP value of 0 mg/dl (X) for which an IBD is not suspected.

$$Y_1 = \frac{e^{-4.61 + 2.68 + 0.13 * 15}}{1 + e^{-4.61 + 2.68 + 0.13 * 15}} = 0.504$$

Figure 7. Model #1.

$$Y_1 = \frac{e^{-4.61 + 2.68}}{1 + e^{-4.61 + 2.68}} = 0.127$$

Figure 8. Model #2.

$$Y_2 = \frac{e^{-4.61}}{1 + e^{-4.61}} = 0.009$$

Figure 9. Model #3.

DISCUSSION

The finding of more than half of the samples positive by means of the molecular method, and *Campylobacter* spp. and *Salmonella* spp. as the most frequent pathogens found, matches a previous study performed at our institution.[10] In that study, the population evaluated was composed of 60/183 (32.8%) pediatric patients, and that explains some of the differences, such as a greater number of *C. difficile* Toxin A/B [15/94 (16%)] compared to the proportion found in the present study [5/76 (6.6%)]. In this study, we decided to exclude *C. difficile* Toxin A/B from group 1 pathogens, since not only the prevalence in our pediatric population is low, but as reported by the 2017 Clinical Practice Guidelines for *C. difficile* Infection in Adults and Children,[14] in patients ≥2 years of age, testing is recommended in the presence of IBD or specific risk factors, such as recent antibiotic therapy.[14]

Regarding the pathogens isolated by culture, of the 21 samples positive for *Campylobacter* spp. by molecular method, 13 (62%) were isolated also by culture (Figure 2). The MIC distributions for the main drugs tested according to EUCAST guidelines,[11-13] showed a percentage of resistance to ciprofloxacin of 77% (10/13) and to tetracycline of 38.5% (5/13). No resistance to erythromycin was found. This finding is partially in line with that of Sasaki *et al.*[15] who, in a study on 151 clinical isolates of *C. jejuni* from patients suffering from enteritis, found no resistance to erythromycin and resistance rates to ciprofloxacin and tetracycline of 46.4% and 23.8%, respectively. On the other hand, in a report by García-Fernández *et al.* [16] on 647 *Campylobacter* spp. strains, similar resistance rates were observed for *C. jejuni* to ciprofloxacin (76%), but higher rates for tetracycline (61%) and erythromycin (7%).

With respect to *Salmonella* spp., from all the 23 samples positive by the molecular method were also recovered isolates by cul-

ture (Figure 2). The MIC distributions (Figure 4) showed 52.2% (12/23) of isolates resistant to amoxicillin-clavulanic acid, 8.7% (2/23) to ciprofloxacin, 4.3% (1/23) to cotrimoxazole and 100% to both gentamicin and amikacin, irrespective of MIC value, as for EUCAST Expert Rules v 3.2.[17] The resistance rate to ciprofloxacin found in this study is much lower than that found by Pitti *et al*.,[18] around 50%, even more for cefotaxime, ranging from 40% during 2012-2016 to 10% during 2017-2021, while no resistance to cefotaxime was found in the present study. This is possibly due to the population evaluated in the present study. Concerning amoxicillin-clavulanic acid, the MIC distribution suggests a double population of isolates, half susceptible and the other half resistant. This is an interesting result, and in the future, an *ad hoc* clonality assessment will be performed. The MIC distributions found in this study substantially match those reported in the MIC and zone diameter distributions and ECOFFs by EUCAST.[19] The local antimicrobial susceptibility patterns found for the two most frequent pathogens during the time period considered allow the Pediatrician to predict with a good chance the more appropriate therapy well before the result of the antibiogram from stool culture.

The comparison of proportions performed between results by molecular method and culture on culturable pathogens showed a significantly greater number of identifications by the former. An even more marked difference was found if positive results for enteric viruses were considered (data not shown). This result means that the information provided by the two methods is quite different, in both time and knowledge and is in line with that reported in our previous study,[10] in which 39 pathogens were identified by molecular method compared to 29 by culture, with a significant difference. This finding is of particular interest, because getting confirmation of the infectious etiology of diarrhea affecting a pediatric patient and also identifying the pathogen in a few hours, allows the Pediatrician to administer a more targeted antibiotic therapy if necessary and reduces the need for imaging study, shortening length of stay and time to discharge.[7,8]

Among the clinical data available at presentation, in this study, we found that the presence of blood/mucus in stools is independently associated with a greater probability of having a positive result for group 1 pathogens (Table 4). This is consistent with the knowledge that bacterial diarrhea is associated with more severe clinical pictures with blood and mucus.[2] Concerning the lack of independent association of vomiting or fever with group 1 pathogens (Tables 3 and 4), that can be explained by the presence of these two signs in both bacterial and viral diarrhea. [1]

The finding of a higher median value of CRP in patients positive for group 1 pathogens (Table 4) is supported by other studies, such as that of Marcus *et al*.,[20] in which, on 44 children suffering from gastroenteritis, higher levels were associated with a greater likelihood of a positive bacterial culture. Also, Borgnolo *et al*.,[21] in a study on 53 children with bacterial infection *vs* 35 with viral infection, found higher CRP values in the former group. Finally, in our previous study,[10] we found significantly higher median CRP values in patients suffering from bacterial gastroenteritis. In particular, the AUC of 0.701 found in this study (Figure 5) suggests a fair discrimination power between groups 1 and 2 as a stand-alone test.

Concerning the regression models built to provide an estimate of the probability of a molecular test positive for group 1 pathogens given the presence of some of the variables considered, it is clear that according to our data, especially in the presence of blood/mucus in stools and when IBD is not suspected, the probability is a function of the inflammatory state expressed by CRP value. Indeed, as reported in Model #1, even if not enough discriminating per se, within the group of patients suffering from diarrhea with blood/mucus in stools, without the suspicion of an IBD, values of CRP ≥15 mg/dL drive up the probability to more than 50%. Conversely, the only presence of a diarrheal illness, without blood/mucus in stools, associated with a CRP value below the lower limit of detection, corresponds to a conditional probability of 0.9% to have a molecular test positive for group 1 pathogens (Model #3). As evident by Table 4, the suspicion of an IBD further reduces the likelihood of diarrhea caused by group 1 pathogens in our pediatric population, since it represents a risk factor for *C. difficile* infection.[22]

This study has limitations. The variables included in the study are not the only ones that can be used to build such a prediction model, and the sample size is limited. Another study with more patients and a wider range of variables will be the subject of a future project.

CONCLUSIONS

The results proposed in this paper can be of help in hospital settings without the availability of a stool multiplex PCR assay to estimate the probability of bacterial diarrhea in a pediatric patient.

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