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Comparative Evaluation of Multiplex PCR And Antigen Testing for Rotavirus Diagnosis in Symptomatic Children Aged 0 To 5 Years in Douala, Cameroon

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ABSTRACT

Background: Rotavirus is a common cause of acute gastroenteritis in children under 5 years of age. Diagnosis is often based on antigen tests, but multiplex PCR could offer better accuracy. It would therefore be useful to evaluate the value of multiplex PCR in the diagnosis of rotavirus infections in symptomatic children aged 0 to 5 years, with the aim of improving diagnostic strategies and the management of rotavirus infections.

Methodology: This cross-sectional and analytical study was conducted between September 2023 and September 2024. The participants, children aged 0 to 5 years presenting symptoms of gastroenteritis were recruited in two hospitals in Douala: the Douala Gyneco-Obstetric and Pediatric Hospital and Laquintinie Hospital. After informed consent obtained from the participants, samples were analysed using antigenic tests and multiplex PCR to detect the presence of rotavirus. The data were processed with SPSS and STATA software for statistical analyses.

Results: Of the 75 samples analysed, 16% (12/75) were positive for rotavirus with antigen tests and 34.67% (26/75) with multiplex PCR. Co-infections with other germs were found in 17.33% (13/75) of cases, the most frequent pathogens being *Shigella spp* (5.33%) and *Adenovirus* (6.67%). The antigen test showed a sensitivity of 38.5% and a specificity of 95.9%. The ROC curve revealed that multiplex RT-PCR outperformed the antigen test with an AUC of 0.765 compared to 0.593 for the antigen test. The agreement between the two tests was low (Kappa value = 0.394). Factors associated with rotavirus include number of stools per day (OR = 3.09; p = 0.023), nature of stool (OR = 6.00; p = 0.001), and vaccination (OR = 1.68; p = 0.009). Logistic regression showed that vaccinated children were 3.579 times more likely to be rotavirus positive (p = 0.015).

Conclusion: Multiplex PCR was found to perform better than antigen testing in the diagnosis of rotavirus infections. Its implementation in hospitals should be encouraged to optimize the detection of rotavirus and/or other pathogens responsible for diarrhoea.

Key words: Diarrhoea, gastroenteritis, rotavirus, RDT, multiplex RT-PCR.

Background

Rotavirus diarrhoea is the most widespread cause of severe diarrhoea with dehydration in children; it represents 38% of diarrhoea cases, with 215,000 deaths of children under five years of age per year [1–3].

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Group A rotaviruses, made up of numerous strains, cause the majority of infections with approximately 90% of human infections, causing watery, non-bloody diarrhoea and generally disappearing within seven days [4]. Rotaviruses constitute a public health problem, not only in developing countries where all children are infected before the age of two to three years, but also in developed countries where hygienic conditions are better [5–7]. In developing countries, these infections remain significant due to certain factors such as dysfunctional health systems, malnutrition, insufficient budgets allocated to health, insufficient equipment and qualified personnel for better care of these children [6, 8]. In Cameroon, the prevalence of rotavirus varies, depending on studies carried out in different regions: 30% in the Centre [9], 54.6% in the Littoral [8], 46.5% in the Far North and 33.9% in the North-West [10]. Rotavirus-related mortality among children under five years of age is estimated to be 5,825 deaths per year in Cameroon [11]. Different techniques exist for the detection of viruses in stools, including: viral detection by cell cultures, the search for viral genomes, the search for viral antigens and the search for resistant viruses. The diagnosis of rotavirus diarrhoea in Cameroon may be insufficient because it is not integrated into the routine laboratory diagnostic process; few health facilities carry out rotavirus screening tests [10]. Immunochromatographic or rapid diagnostic tests (RDTs) are easy-to-use screening tools in hospitals, but are not always available [12]. Real-time PCR (RT-PCR) is a relatively rapid and reliable method that can be used for the simultaneous detection and quantification of viral infections [16], and Multiplex RT-PCR makes it possible to detect several pathogenic agents or various strains in a single step, making it possible to identify the different aetiologies of diarrhoea and improve their management [17, 18]. Choosing which of these techniques to implement routinely would be useful in hospitals. This study aims at improving diagnostic strategies for gastrointestinal infections in children, by comparing the performance of multiplex RT-PCR and antigen tests. To reach our goal, we will determine the epidemiological and clinical characteristics of the study population, estimate the prevalence of rotavirus infections among symptomatic children aged 0 to 5 years in Douala, compare the diagnostic performance of multiplex PCR (A.I.I. Screen Real-TM Sacace) to that of the BiolineTM Rotavirus antigen test in the diagnosis of rotaviruses, and identify the factors associated with the presence of rotavirus in these children.

METHODS

This was a cross-sectional and analytical study. We included hospitalized children aged 0 to 5 years (0 to 60 months) and/or consulting in the pediatric department for gastroenteritis. The study was conducted from September 1, 2023 to September 1, 2024 in Douala at the Douala Gyneco-obstetric and Pediatric Hospital (DGOPH) and Laquintinie Hospital (LQH). Study participants were recruited during daily pediatric consultations in pediatric departments of each hospital. During these consultations, particular attention was paid to children presenting symptoms compatible with rotavirus infections, such as episodes of acute diarrhoea. All samples were analysed at the DGOPH shortly after collection.

Rapid rotavirus diagnostic testing was carried out with a rapid diagnostic test (RDT): *TDR Bioline^{TM} Rotavirus Abbott* for the detection of Group A rotaviruses in stool. This kit uses two kinds of antibodies and a solid-phase immunochromatographic process to detect group-specific proteins. The testing procedure was carried out following the manufacturer's instructions available in the kit. PCR was carried out using the *A.I.I. Screen Real-TM* kit by *Sacace Biotechnologies/Italy* which comes along with its *DNA/RNA Prep* kit for nucleic acid extraction and an amplification kit. This multiplex PCR kit can detect eight intestinal infectious organisms: *E. coli, Salmonella spp., Shigella spp., Campilobacter spp., Norovirus, Astrovirus, Adenovirus,* and *Rotavirus*. The *Amplix 12* automatic extractor was used for nucleic acid extraction and the *DTlite 4S1* thermal cycler was used for amplification. Result analysis were performed following the manufacturer's instructions.

Data was collected on a Microsoft Excel VBA form. The consistency and quality of the data was checked using the Excel filter. Firstly, a flat sorting was carried out on each variable to observe the varied united distributions. Cross-tabulations, P-value, Odds ratio, sensitivity and specificity were obtained from SPSS v24.0. Binary logistic regressions was done using STATA 16. The description of the qualitative variables presents the numbers and percentages while that of the quantitative variables allows us to have the mean, standard deviation, minimum, maximum and interquartile range. The contingency tables of the qualitative variables made it possible to highlight Odds Ratios followed by their 95% confidence interval. The comparison of the proportions obtained in the cross tables revealed the tests of independence of the variables (Chi-square). The statistical significance threshold used was $p<0.05$.

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We obtained clearance from the Institutional Research Ethics Committee of the Faculty of Medicine and Biomedical Sciences (FMBS) Yaoundé, and that of the Institutional Research Ethics Committee for human health of the Douala Gyneco-Obstetric and Pediatric Hospital (DGOPH). We also obtained authorizations for data collection and analysis from the Director of the Douala Gyneco-Obstetric and Pediatric Hospital and Laquintinie Hospital (LQH). We obtained signed informed consent from parents or the legal guardians of all patients that gave their samples.

RESULTS

Epidemiological and clinical characteristics of the study population

A total of 75 patients were recruited and provided stools for this study. The M/F sex ratio was 1.02. The average age was 32.22 (±19.73) months. The primary school level was the most represented among parents/guardians 48% (Table 1). The majority (85.33%) of children were vaccinated against Rotavirus (Table 2). Among the patients, 27 (36%) presented vomiting, 50 (66.67%) were hospitalized, and 18 (24%) presented moderate dehydration. The most frequent type of stool was liquid (54.67%). Close to half (48%) of the children had moderate malnutrition, and 3 patients (4%) had a positive HIV immunological status (Table 3).

Table 1: Epidemiological characteristics of the study population

Variable	**Population (N=75)**	**Percentage (%)**
Sex		
Males	38	50.67
Females	37	49.33
Age (in months)		
[0-1[	1	1.33
[1-24[	27	36.0
[24- 60]	47	62.67
Parents' educational level		
Primary	36	48.0
Secondary	23	30.67
University level	16	21.33

Table 2: Distribution according to background and lifestyle

Variables	**Population (N=75)**	**Percentage (%)**
Type of beverage consumed		
Mineral water	26	34.67
Tap water	49	65.33
Borehole water	0	0.0

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Well water	0	0.0
Vaccination (Rotarix®)		
Yes	64	85.33
No	11	14.67

Table 3: Distribution according to clinical profile

Variables	**Population (N=75)**	**Percentage (%)**
Clinical components		
Vomiting	27	36.0
Temperature (°C)		
[37° - 37,4]	11	14.67
[37,5 – 38,5]	31	41.33
>38,5	33	44.0
Hospitalisation		
Yes	50	66.67
No	25	33.33
Dehydration		
Yes	18	24.0
No	57	76.0
Classification of dehydration (n=18)		
Mild	0	0.0
Moderate	18	100
Number of stools per day		
3 stools	38	50.67
[4 - 5] stools	30	40.0
≥ 6 stools	7	9.33
Stool consistency		
Liquid	41	54.67
Glaireous	20	26.67

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Loose	14	18.67
Nutritional status		
Normal	39	52.0
Moderate malnutrition	36	48.0
HIV status		
Positive	3	4.0
Negative	72	96.0

Prevalence of Rotavirus

Out of 75 samples tested, 16% (12/75) were positive for rotavirus on RDT, while 34.67% (26/75) were positive for rotavirus on multiplex RT-PCR (Table 4). We observed 13 cases of co-infection with rotavirus from multiplex PCR results. Co-infection with *Shigella spp* was most common and all other co-infections were equal in frequency (Table 5).

Table 4: Estimating the prevalence of rotavirus infections

Variables	**Population (N=75)**	**Percentage (%)**
Antigen test		
Positive	12	16.0
Negative	63	84.0
Molecular test		
Positive	26	34.67
Negative	49	65.33

Table 5: Germs found in co-infections with rotavirus

Variables	**Population (N=75)**	**Percentage (%)**
Co-infection (n=13)		**17.33**
Shigella spp	04	5.33
Adenovirus	01	1.33
Salmonella spp	01	1.33
Norovirus	01	1.33
Astrovirus	01	1.33
Campylobacter spp	01	1.33

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Adenovirus, Norovirus	01	1.33
Astrovirus, Adenovirus	01	1.33
Campylobacter spp, Adenovirus	01	1.33
Salmonella spp, Adenovirus	01	1.33

Comparison of the diagnostic performance of multiplex PCR (A.I.I. Screen Real-TM Sacace) to that of the Rotavirus antigen test (Bioline^{TM} Rotavirus)

It appears at the end of our study that the sensitivity of the antigen test is 38.5%, and its specificity is 95.9% (Table 6). The ROC curve reveals that multiplex RT-PCR is more sensitive than antigen tests (Figure 1). The Multiplex PCR test outperforms (AUC=0,765) the antigen test (Table 7). Moreover, our results also show that our two tests have low agreement (Cohen's Kappa value = 0.394) in the diagnosis of rotavirus (Table 8).

Table 6: Multiplex PCR (Sacace) and the antigen test (Bioline^{TM} Rotavirus) performance in the diagnosis of rotavirus

	Molecular test		***P* value**	**Sensitivity**	**Specificity**
	Positive (%)	Negative (%)			
Antigen test					
Positive	10 (13.33)	2 (2.67)	**0.000111**	**0.385**	**0.959**
Negative	16 (21.33)	47 (62.67)			
Total	26 (34.67)	49 (65.33)			

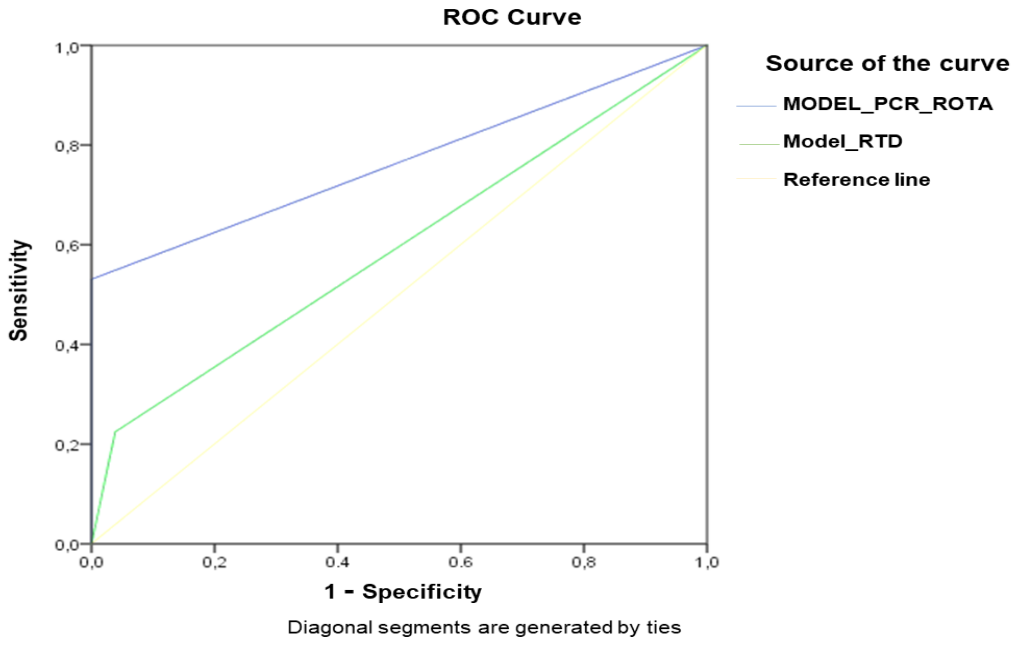

Figure 1: Sensitivity comparison between Multiplex PCR and RDT

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Table 7: Performance of multiplex PCR compared to antigen test

Calculation of the AUC index					
	AUC Index	Standard error	Asymptotic significance	Asymptotic confidence interval (95%)	
				Lower limit	Upper limit
RT-PCR Multiplex	**0.765**	0.053	0.000168	0.661	0.869
RDT	0.593	0.066	0.187	0.463	0.723

Hint: AUC = Area Under the Curve

Table 8: Concordance between Multiplex PCR and antigen test

		RDT			**Cohen's Kappa**			
		Negative	Positive	Total	Value	Asymptotic standard error	Approximate T	Approximate significance
Multiplex PCR	Negative	47	2	49	**0.394**	**0.107**	**3.865**	**0.000111**
	Positive	16	10	26				
	Total	63	12	75				

Clinical and epidemiological factors associated with the presence of rotavirus

Vaccinated children were 3.579 (1.6321-5.1824) times more likely to be rotavirus positive (p=0.015), while stool consistency indicated a 1.994 (1.009-3.941) times likelihood of being rotavirus positive (Table 9).

Table 9: Binary logistic regression of factors associated with rotavirus

Variables	Adjusted OR (95% CI)	Adjusted *p* value
Age	0.854 (0.565-1.288)	0.452
Sex	0.936 (0.329-2.662)	0.902
Temperature	0.241 (0.090-0.641)	0.004
Hospitalisation	2.811 (0.564-14.008)	0.207
Dehydration	2.073 (0.538-7.983)	0.289
Number of stools per day	1.867 (0.752-4.634)	0.178
Stool consistency	**1.994 (1.009-3.941)**	**0.047**
Vaccine	**3.579 (1.632-5.182)**	**0.015**

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DISCUSSION

Epidemiological and clinical characteristics of the study population

We obtained 75 diarrheal stool samples from children aged 0 to 5 years suffering from gastroenteritis. This relatively low number may be as a result of one of our selection criteria (number of daily stools ≥ 3), and the number of health facilities in which recruitment took place. The average age and sex ratio in our study are consistent with that of Levoa Eteme et al. [9] in a study carried out in Yaoundé (Cameroon) in 2015, and other studies carried out in other countries [19-21]. This can be explained by the fact that male children tend to develop greater muscle mass due to the action of sex hormones. These hormones impact the balance of cytokines and T-helper lymphocytes, thereby reducing resistance to infectious diseases [22]. In addition, micronutrient deficiency (notably zinc and vitamin A) constitutes a factor favouring the occurrence of gastroenteritis.

The most represented educational level among parents/guardians was primary (48%). Mungyeh et al. [23] as well as Mahamadou et al. [24] obtained similar results in their studies carried out in Yaoundé and Bamako respectively. The parents' low educational level could explain the lack of understanding or non-application of hygiene and sanitation rules.

Moderate malnutrition was found in 48% of infected children which is consistent with what Djikoloum et al. [21] obtained in Chad. A large number of micronutrients, such as minerals and vitamins, as well as some macronutrients, such as certain amino acids, cholesterol and fatty acids have been shown to exert a very significant and specific impact on the immune system activity [25]. Diarrhoea, which is one of the main causes of malnutrition in children under five years old causes dehydration due to loss of fluids and electrolytes from the body [26]. Infants have relatively greater fluid requirements due to increased insensible fluid loss [26].

Prevalence of Rotavirus

The prevalence of rotavirus in our study (34.67%) is not too far from the 30% obtained by Levoa Eteme et al. [9] in 2016 in the Central region of Cameroon, as well as that carried out in Benin (39.8%) by Agbla Michel et al. [19] in 2020. But it is lower than the 54.6% obtained by Ghapoutsa et al. [8] in 2021 in the Littoral region of Cameroon. Other studies carried out in countries adjacent to Cameroon show similar results to ours [27,28]. Our relatively high prevalence can be explained in part by a number of socio-environmental conditions including difficult access to drinking water, and the poor economic situation of parents which has a negative impact on living standards and sanitary conditions.

Our results also highlight the association of certain viruses and bacteria with acute gastroenteritis in young children. These associations have also been reported in studies carried out in other Central Africa and in other parts of the world [17,21,27]. The frequency of co-infection of rotavirus with other enteric pathogens found in our study was 17.33%. *Shigella spp* was the most identified bacteria in co-infections while Adenovirus was the most identified virus. This is consistent with what Ghapoutsa et al. [12] obtained from the entire Littoral region (Cameroon), explaining that the presence of other co-infecting enteric pathogens increases rotavirus replication in the host intestine.

Comparison of multiplex PCR (Sacace Biotechnologies) and the antigen test (TDR Bioline^{TM} Rotavirus Abbot) in the diagnosis of rotaviruses

We obtained a sensitivity of 38.5% and a specificity of 95.9%, as well as a negative predictive value (NPV) of 75% and a positive predictive value (PPV) of 83% for the antigen test. Thangjui et al. [30] found a sensitivity of 44.8% and a specificity of 97.7%, which is not too different from ours, for the Vikia® RDT. Several studies, including ours, have demonstrated the presence of cross-reactions (false positives) between rotavirus and other pathogens, notably norovirus which is also an RNA virus [31–34]. Samples that were positive with the antigen test had a high viral load with multiplex PCR. The antigen test appears to be most effective in individuals with a high viral load. This was also noticed in a study carried out by the WHO in 2019 [35]. Our results also show that positive antigen test results are higher in patients who produced a high number of stools

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(≥ 6). Multiplex PCR had a better sensitivity and specificity than rapid diagnostic test. It has a lower limit of detection (LOD) and a lower limit of quantification (LOQ) than antigen tests. In addition, multiplex PCR makes it possible to highlight co-infections between different pathogens responsible for diarrhoea in one step, avoiding the need to conduct an individual test search for each of them and thus offering rational and targeted guidance for therapeutic management [36]. Multiplex PCR permits the extraction and amplification of a specific gene sequences, making the result more reliable. The antigen test makes detects the presence of antigens, which may or may not be present in sufficient quantities, and which may be close to the antigens of another pathogen, hence may be responsible for cross-reactions (false negative or false positive) [36, 37]. Unlike multiplex PCR, the antigen test is easy to use with easy implementation, does not require specific premises or special training and has a rapid turnaround time.

Factors associated with the presence of rotavirus

According to the CDC, the factors generally associated with rotavirus are liquid stools, fever (≥39°C) and vomiting, correlated with dehydration [38]. Variables significantly associated with rotavirus infection in our study were stool consistency and vaccination. Contrary to the data in the literature, which describe the emission of liquid stools and an absence of mucus and/or blood in patients with rotavirus gastroenteritis [2], the mucus-like stools were those which had the highest number of positive results. These results suggest the need to search for rotavirus in stool with mucus. On the other hand, they suggest it is less likely to find rotavirus in loose stools. Studies have shown that there is a gradient in rotavirus vaccine effectiveness between developing and developed countries. In developed countries this gradient is higher. Indeed, poor hygiene conditions, malnutrition, and lack of access to drinking water, as well as the presence of co-infection with other pathogens responsible for gastroenteritis contribute to reducing vaccine effectiveness [39]. Malnutrition could impair the development of a protective immune response after vaccination, which can favour rotavirus infection in children. Poor hygiene and sanitation conditions, as well as a lack of drinking water, expose the body to other pathogens responsible for gastroenteritis, which further reduce the immune response of the vaccine [39,40]. In our study, all vaccinated children were positive for rotavirus. The vaccines used (Rotarix® and RotaTeq®) were administered orally. The induced immunity is secretory with production of IgA type immunoglobulin [11,23,39], which do not protect for a long period. Nevertheless, it prevents the development of severe forms of the disease [39]. This allows us to suggest a more in-depth study on this aspect, in order to implement more effective vaccines and vaccination programs.

CONCLUSION AND RECOMMENDATIONS

The present study which had as objective to evaluate the interest of multiplex PCR in the diagnosis of rotavirus infections in symptomatic children aged 0 to 5 years in Douala, highlights that the prevalence of rotavirus is significant in children with acute diarrhoea. Male children and those aged 36 months and over are most at risk. This viral infection remains a major cause of morbidity in this population. The observed prevalence reflects the importance of continuous epidemiological surveillance to better understand the extent of the disease and its impacts on public health. Regarding the performance of diagnostic tests, multiplex PCR performs better than antigen testing in the diagnosis of rotavirus in symptomatic children, with better sensitivity and specificity. This method should be favoured in clinical environments where accurate detection of rotavirus is essential for adequate management. Several factors associated with rotavirus infection have been found, such as the clinical profile and vaccination. These elements must be taken into account in the development of prevention strategies, particularly in the most vulnerable populations, in order to reduce the transmission and incidence of infection.

Limitations of the study

Concerning the molecular test, we did not mention the targeted sequences of the gene of interest, because it is a commercial kit and the company has not communicated on this. The limits of detection and of quantification (LOD and LOQ) of the multiplex PCR have not been determined or mentioned either.

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