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**DETECCIÓN Y GENOTIPIFICACIÓN DE ROTAVIRUS EN
PACIENTES CON GASTROENTERITIS AGUDA**

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**DIARRHEA BY ROTAVIRUS IN A REGIONAL PERUVIAN HOSPITAL: DETERMINATION OF
CIRCULATING GENOTYPES.**

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ABSTRACT

Background: Gastroenteritis by rotavirus is responsible for approximately 810 annual deaths/year in children under 5 years in Peru and emerging rotavirus genotypes have led to concerns regarding cross-protection by the vaccines available. Moreover, there are no reports on the molecular-epidemiology of rotavirus diarrhea in Peru

Methodology: A total of 131 stool samples were obtained from children under 5 years old hospitalized from January 2010 to December 2012 in the Hospital Regional de Cajamarca, Peru. ELISA and RT-PCR techniques were performed for rotavirus detection. G and P typing of rotavirus-positive samples were obtained by semi-nested multiplex RT-PCR and sequencing was performed to confirm the PCR results.

Results: Of the 117 samples available, 18.80% (22/117) tested positive for rotavirus by ELISA and 35.90% (42/117) by RT-PCR. Among the G-genotype identified, G9 in 35.71% (15/42) and G12 in 33.33% (14/42) were the most prevalent. With the most common combination being G12/P6 in 23.81% (10/42).

Conclusions: A high prevalence of the G12/P6 genotype was detected. It is know that this genotype is not covered by the current vaccines available. More in depth studies are needed to know the current rotavirus genotypes presents in Peru.

Key words: Rotavirus, viral genotypes, epidemiology, acute gastroenteritis, Peru.

INTRODUCTION

Rotavirus is the most important cause of severe gastroenteritis accompanied by acute diarrhea in young children worldwide¹. In Peru, human rotaviruses are responsible for approximately 810 annual deaths in children under 5 years old².

The genus Rotavirus belongs to the family of Reoviridae which contains 11 segments of double-stranded RNA that is located inside a triple-layered virus particle. Rotaviruses are classified into seven serogroups (A to G), based on the characteristics of the VP6 membrane protein and divided into G and P genotypes, according to the genetic and antigenic diversity of the two outer capsid proteins VP7 and VP4, respectively^{3,4,5}. At least 27 G types and 35 P types have been identified and the most prevalent genotypes in humans are G1P [8], G2P [4], G3P [8], G4P [8], and G9P [8]^{6,7}. However, in the last few years Argentina, Chile and Brazil have reported new genotypes that have apparently spread in Latin America. Knowledge of their distribution, including detection of emerging genotypes, is crucial for rotavirus vaccination programs^{8,9,10}.

The major symptoms of rotavirus gastroenteritis in children include watery diarrhea, vomiting, respiratory symptoms, and fever^{11,12}. Laboratory diagnosis of rotavirus infection is usually performed by antigen detection, using latex agglutination or more recently enzyme immunoassay (ELISA). However, in multiple studies both techniques have shown variable sensitivity and specificity with a risk of misdiagnosis^{13,14}. Polymerase Chain Reaction (PCR) has become the preferred method for human rotaviruses detection as well as genotype characterization in epidemiologic studies^{15,16}.

In 2009, two rotavirus vaccines were introduced by the Peruvian National Immunization Program. A live attenuated vaccine called *Rotarix* specific for the G1P[8] genotype and it is administered in a 2-dose series in infants and children. The second vaccine is *Rotateq*, a

live oral pentavalent formula for serotypes G1, G2, G3, G4 and G6¹⁷. Both vaccines have shown to be safe, providing more than 70% and 90% protection against any rotavirus diarrhea and severe diarrhea, respectively¹⁸.

There are no studies on the current rotavirus genotypes circulating in Peru, and the emergence of new strains has led to new concerns regarding cross-protection by the present vaccine formulations. Hence continuous surveillance of rotaviruses for the investigation of the emergence of new strains is required, including the collection of baseline data aimed to evaluate the vaccines commercially available.

In this study we assessed the prevalence of rotavirus infection and genotypes using ELISA and PCR amplification of viral nucleic acid derived from stool specimens of children under 5 years old from the Regional Hospital of Cajamarca in Peru.

MATERIAL AND METHODS

Study population and collection of specimens

A total of 131 stool samples were obtained from children under 5 years old hospitalized with acute gastroenteritis from January 2010 to December 2012 in the *Hospital Regional de Cajamarca*, Peru. Samples were collected between 1 to 3 days after disease onset. Clinical data were registered by physicians in the hospital. Acute gastroenteritis was defined as the occurrence of diarrhea lasting less than 14 days along with symptoms such as vomiting, fever, dehydration, and abdominal pain according to the European Society of Paediatric Infectious Disease¹⁹. Fever was defined as an axillary temperature of ≥ 37.5 °C. Nutritional status was based on weight-for-age Z scores (WAZ), calculated using the least mean square method and the 2000 CDC Growth Reference²⁰. Dehydration status was established according to World Health Organization (WHO) criteria²¹. Fecal samples were transported at 4°C to the laboratory of the Microbiology Department of *Dirección Regional de Salud de Cajamarca*, Peru. The samples were stored at -20°C, until be sent to be processed in the Research Center for Health Sciences, *Universidad Peruana de Ciencias Aplicadas*, Lima, Peru.

This study was approved by the Ethics Committee of the *Universidad Peruana de Ciencias Aplicadas* and an informed consent was signed by the parents before sample analysis was performed.

Rotavirus detection by ELISA

Rotavirus antigen (group A rotavirus-specific VP6 proteins) was detected using an enzyme-linked immunosorbent assay (ELISA; RotaClone, Meridian Bioscience).

Extraction of dsRNA

Rotavirus-positive fecal suspensions were used for viral double-stranded RNA (dsRNA) extraction using the QIAamp viral RNA kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. Viral dsRNA obtained after extraction was stored at -20°C until use.

Amplification of the Rotavirus VP4 and VP7 genes. Rotavirus dsRNA was reverse transcribed using the OneStep RT-PCR kit (Merck4Biosciences, Germany). After denaturation of the RNA at 80°C for 5 min, the RT-PCR comprised a reverse transcription step at 50°C for 30 min.

The primers used for amplification of VP7 gene sequences have been previously described by Gouvea et al.²², Griffin et al.²³, and VP4 gene sequences by Gentsch et al.²⁴. The PCR mixture was 50 µl, distributed as follows: 25 µl of enzyme mix (Taq polymerase, 2.5 mM MgCl₂, 15 mM Tris / HCl pH 8.3, 50 mM KCl, 200 µM of each deoxynucleotide) (Kapa Biosystem, USA), 20 pmol of each primer (Macrogen, Seoul, Korea), and 5 µl of cDNA.

The PCR conditions were: 95°C for 10 min, followed by 45 cycles of 94°C for 30 sec, 52°C for 45 sec and 72°C for 1 min, with a final elongation of 10 min at 72 °C. The amplification products were analyzed by gel electrophoresis on 2% agarose (FMC, Rockland, ME) gel containing 3 mg/L ethidium bromide. Sequencing was performed to confirm the PCR results. Amplified products were recovered from the gel, purified (SpinPrep™ Gel DNA Kit, San Diego, USA) and sent for commercial sequencing service (Macrogen, Seoul, Korea).

G and P genotyping by semi-nested multiplex RT-PCR

Rotavirus dsRNA was reverse transcribed using the OneStep RT-PCR kit (Merck4Biosciences, Germany). G and P typing of the strains was performed by semi-nested multiplex PCR assays using consensus and type-specific primers^{22,23,24}.

The PCR conditions were: 95°C for 10 min, followed by 45 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, with a final elongation of 10 min at 72 °C. The amplified products were analyzed, recovered and sequence as mentioned above to confirm the PCR results.

Data analysis

Statistical significance was established using the Fisher exact test. The differences were considered as significant with a p-value<0.05. Statistical analyses were performed with SPSS software (Microsoft SPSS-PC+, v.15.0; SPSS, Chicago, IL, USA).

RESULTS

Study Population

Of the 131 samples collected from children under 5 years and hospitalized for acute gastroenteritis, 14 samples were excluded from the protocol analysis because they failed to meet the inclusion criteria. Of the remaining 117 samples, 22 (18.80%) tested positive for rotavirus by ELISA and 42 (35.90%) were positive for rotavirus gene amplification by RT-PCR (table 1), indicating the latter as a more suitable assay for rotavirus diagnosis.

Among all the positive rotavirus samples, a higher distribution of cases [45.25% (19/42)] was observed in children older than 18 months (table 2). Of all the rotavirus-positive cases, 35.71% (15/42) received mixed lactation, 30.95% (13/42) only formula, and 23.82% (10/42) exclusive breastfeeding. In addition, 26.19% (11/42) of the patients had some degree of malnutrition.

The most common symptoms of the patients with a positive rotavirus sample were fever in 64.29% (27/42) and vomiting in 57.15% (24/42). In this group mild diarrhea was observed in 42.86% (18/42), moderate diarrhea in 26.20% (11/42) and severe in 19.04% (8/42). On the other hand, one third of rotavirus cases presented mild to moderate dehydration and only 1 case of severe dehydration was observed. During hospitalization and using only clinical criteria, 42.86% (18/42) of patients received common antimicrobial drugs including ampicillin, amikacin, chloramphenicol and cephalexin (table 3).

Rotavirus detection and genotyping

ELISA rotavirus detection showed a sensitivity of 38.10% and a specificity of 92.00% using the RT-PCR as a reference standard; Also a positive and negative predictive values of 72.73% and 72.63% respectively were obtained with a 95% confidence interval (C.I.) (table 4).

The amplification of the VP7 and VP4 genes by a semi-nested multiplex RT-PCR showed the presence of the following G genotypes: G9 in 35.71% (15/42) and G12 in 33.33% (14/42) were the most prevalent, while G3 (15.00%), G4 (5.00%), G1 (2.50%) and G2 (2.50%) were present to lesser frequencies. No G8 was detected. (Table 5).

Regarding VP4 amplification for the P genotypes, the most frequent strains were P6 (59.38%) and P8 (34.38%) while P4 was found in only 2 cases (6.25%). Complete rotavirus genotyping was achieved in 71.43% (30/42) of samples, G12/P6 being the most common genotype in 23.81% (10/42), followed by G9/P6 in 19.05% (8/42) and G9/P8 in 11.90% (5/42) of patients.

DISCUSSION

Rotavirus gastroenteritis is responsible for 40% of the hospitalizations of children worldwide¹. In Peru, the Epidemiologic Surveillance report of 2008 showed that rotavirus was detected in 35.80% of all acute gastroenteritis in pediatric patients under 5 years old. Higher incidences were correlated with children older than 18 months while the most vulnerable population was found those 11 months old or younger². In this study, prevalence of rotavirus was 18.80% (22/117) and 35.90% (42/117) using ELISA and RT-PCR respectively. Approximately, one third of all the children enrolled were between 18 months and 5 years of age and 45.25% (19/42) of them had had a rotavirus-positive test, consistent with previous studies.

Polymerase chain reaction is used widely for rotavirus detection and has become the most popular method for human rotavirus genotyping epidemiology studies^{14,15,25}. Different studies have reported a sensitivity ranging from 84.8-99% and a specificity of 73.3-99.7% with ELISA applying different laboratory assays as reference standards (virus isolation, electronic microscopy, polyacrylamide gel electrophoresis)^{13,27,29}. Using RT-PCR as a confirmatory test for rotavirus detection, we found that ELISA has a very low sensitivity (38.10%) while having a relatively high 92.00% specificity (92.00%).

Rotavirus gastroenteritis is characterized by watery diarrhea, which normally last about 5-6 days, associated with fever and vomiting in 50% and 75% of the cases, respectively²⁸. In our series, fever was the most common symptom, present in 64.29% (27/42), followed by vomiting in 57.15% (24/42) of patients. On admission 42.86% (18/42) of all rotavirus-positive cases presented mild diarrhea and 45.24% (19/42) were found moderate to severe

diarrhea. Moreover, 42.86% (18/42) of the rotavirus cases received antibiotics on hospitalization using only clinical criteria.

Rotavirus genotype surveillance is critical for understanding the efficacy of vaccination, and different behaviors of dominant strains have been reported in the last 20 years in several Latin American countries²⁹. As an example, during a 20-year surveillance period in Brazil, G1 was the most prevalent type during 1986, emerging again in 1993, 1998 and in 2003. G3 became the dominant strain during 1987-1992 and G9 peaked in 1999-2002 and 2005. Finally, G9 was the dominant type in 2006; however in 2007 an increase of G2, with both serotypes co-circulating at comparable proportions revealed that rotavirus types may concomitantly prevail in different geographical locations³⁰.

A recent meta-analysis has reported that in Latin America the most common G type detected is G1 (34.2%), followed by G9 (14.6%) and G2 (14.4%). In P types, clearly P8 was the most frequently detected (56.2%), followed by P4 (22.1%) and P1 (5.4%). Overall, the most prevalent G-P type associations were G1P8 (17.9%), G2P4 (9.1%) and G9P8 (8.8%)²⁹. The same study reported that G1 has a prevalence of 61.3% in Peru based on a 12-article review²⁹. However, none of these Peruvian studies were designed to determine rotavirus genotypes and only 2 articles from 1996 described the prevalence of G1, G2, G3 and G4 prevalence. Unfortunately, in Peru there is no rotavirus genotyping epidemiologic surveillance program and since 1996 no further data have been published. In our study, G9 in 35.71% (15/42) and G12 in 33.33% (14/42) were the most dominant rotavirus genotypes, with the most common combination being G12/P6 in 23.81% (10/42), followed by G9/P6 in 19.05% (8/42) and G9/P8 in 11.90% (5/42) of patients. This G9 dominance was observed in a similar study from Chile published in 2012, in which increases in G9 had been reported in the last few years.

However, in this study the predominant combination was G9P8 (76%) followed by G1P8 (6%) and no G12 was detected¹⁰.

Genotyping of VP7 and VP4 could not be performed in 4.76% (2/42) and 23.81% (10/42) of the cases respectively. This is probably because these strands belong to other G and P proteins that have been reported in Latino America in the last few years, such as G5, G10, G11, P9 or other unidentified genes^{10,29}. It is important to mention that we found a high prevalence of the G12 genotypes in our series, being a relatively new genotype in which vaccine efficacy is not fully understood^{10,31}.

In Latin America, the G9 rotavirus serotype has been associated with a significantly longer duration and higher frequency of diarrhea, longer duration of vomiting, increased hospitalization rate and more-severe dehydration⁸. Although this association remains under debate, we found that almost all our G9/P6 patients had moderate to severe diarrhea associated with fever and vomiting, with more severe diarrhea being reported in patients with G3/-, G9/P6, -/P6 and -/P4 (table 5). However, it is important to remark that the present study was not addressed to establish a genotype-clinical association.

Despite the G9/P8 and G9/P6 genotypes not being included in the two vaccine formulas, different studies have demonstrated a reduction in the number of severe cases (65-85%)^{32,33}. This effect could be due to the presence of P6 and P8 antigens or because of a cross-reaction with different G genotypes or other structural antigens¹⁰.

This study presented two limitations. The first involves the need to transport the samples from Cajamarca to Lima, despite to be frozen we can not ruled out possible sample missmanipulation during any part of the process. The second limitation is that it was designed

only for rotavirus detection in the patients stool specimens. Therefore, the presence of other confounders can not be ruled out..

As in other Latin American countries, epidemiologic surveillance programs for rotavirus genotypes are necessary in Peru, especially related to children who could most benefit from these vaccines. This study demonstrated that prevailing rotavirus genotypes in children can dynamically change over time and highlights the need for further investigations to better establish the burden of rotavirus, the impact of the disease and how the efficacy of vaccination programs is affecting.

Authors' contributions: PW and FO conceived the study; PW, FO and JV designed the study protocol; JV was responsible for obtaining funding and laboratory work supervision; PW and FO performed the PCR for rotavirus detection and genotyping; HC carried out the immunoassays. AC was responsible for the clinical assessment, samples collection and database completion. PW, FO, LJ, JR and JV drafted the manuscript; LJ, JV and JR critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. PW and JV are guarantors of the paper.

No author has a conflict of interests.

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Table 1: Comparison of techniques used to identify rotavirus.

DIAGNOSTIC METHOD	ELISA		PCR	
	Frequency	Prevalence (%)	Frequency	Prevalence (%)
Positive	22	18.80	42	35.90
Negative	95	81.20	75	64.10
Total	117	100.00	117	100.00

Table 2: Age distribution of ≤ 5 year old children and those with rotavirus.

Age months	N (%) of Rotavirus positive	N (%) of Rotavirus negative	N (%) total patients
0 – 5	9 (21.43)	12 (16.00)	21 (17.95)
6 – 11	3 (07.14)	19 (25.33)	22 (18.80)
12 – 17	6 (14.28)	22 (29.34)	28 (23.93)
18 and above	19 (45.25)	19 (25.33)	38 (32.48)
Unknown	5 (11.90)	3 (04.00)	8 (06.84)
Total	42 (100.00)	75 (100.00)	117 (100.00)

Table 3: Characteristics of Rotavirus-Positive and Rotavirus-Negative cases.

Characteristics	Rotavirus Positive (N=42), n (%)	Rotavirus Negative (N=75), n (%)
Lactation		
▪ Exclusive breastfeeding	10 (23.82)	23(30.67)
▪ Formula	13 (30.95)	7 (09.33)
▪ Mixed	15 (35.71)	31 (41.33)
▪ Unknown	4 (09.52)	14 (18.67)
Symptoms before hospitalization		
▪ Vomiting	24 (57.15)	38 (50.67)
▪ Fever	27 (64.29)	34 (45.33)
▪ Malnutrition	11 (26.19)	8 (10.67)
Diarrhea		
▪ Mild (1-6)	18 (42.86)	23 (30.67)
▪ Moderate (7-10)	11 (26.20)	8 (10.67)
▪ Severe (≥ 11)	8 (19.04)	24 (32.00)
▪ Unknown	5 (11.90)	20 (26.66)
Degree of dehydration		
▪ No dehydration	27 (64.29)	50 (66.67)
▪ Mild or moderate	14 (33.33)	23 (30.67)
▪ Severe		

1 (02.38) 2 (02.66)

Treatment received during hospitalization

▪ Oral rehydration	15 (35.71)	45 (60.00)
▪ Intravenous rehydration therapy	9 (21.43)	8 (10.67)
▪ Antibiotic	18 (42.86)	22 (29.33)

Table 4: Sensitivity and Specificity of ELISA

		95 % C.I	
		Inferior Limit	Superior Limit
Disease Prevalence	35.90%	27.39%	45.35%
Correctly diagnose patients	72.65%	63.50%	80.29%
Sensitivity	38.10%	23.99%	54.35%
Specificity	92.00%	82.79%	96.71%
Positive Predictive Value	72.73%	49.56%	88.39%
Negative Predictive Value	72.63%	62.36%	81.04%

C.I. Confidence Interval

1 **Table 5: Human Rotavirus genotype distribution by multiplex polymerase chain reaction.**

G type / P type	Frequency (n=42)	Prevalence (%)	Diarrhea				Fever			Vomiting		
			L (17)	M (10)	S (4)	U (11)	Y (28)	N (8)	U (6)	Y (27)	N (9)	U (6)
G1 /—	1	02.38	1				1			1		
G2 /—	1	02.38				1				1		1
G3 /—	6	14.29	2		1	3	3	2	1	4	1	1
G9 /—	2	04.76	1			1	1		1	1		1
G3 /P6	1	02.38		1			1				1	
G4 /P8	2	04.76	1	1			2			1	1	
G9 /P6	8	19.05	1	4	1	2	6	1	1	6	1	1
G9 /P8	5	11.90	3	1		1	3	1	1	2	2	1

2

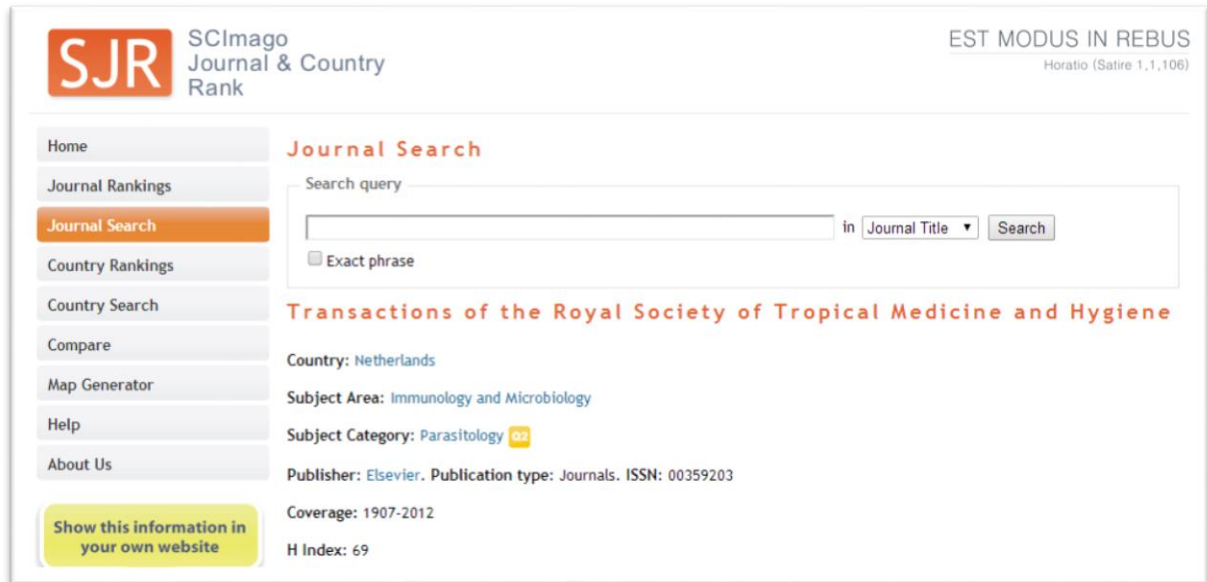
G12 / P6	10	23.81	6	2	1	1	7	2	1	8	1	1
— / P4	2	04.76		1	1		1	1		1	1	
G12 / P8	4	09.52	2			2	3	1		3	1	

3 **L= Mild, M= Moderate, S= Severe, U= Unknown, Y= YES, N= NO**

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JUSTIFICACION

Se optó por esta revista en particular, ya que publica temas relacionados con el estudio desarrollado y, además, se encuentra en el segundo cuartil según el ranking de SCImago para revistas científicas.

PROCESO DE REVISIÓN

Recepción del Artículo a la Revista y asignación a Editor

De: em.trstmh.0.3886bd.2e9db607@editorialmanager.com [em.trstmh.0.3886bd.2e9db607@editorialmanager.com] en nombre de Transactions [journals@rstmh.org]
Enviado: jueves, 16 de enero de 2014 23:19
Para: Juana Del Valle Mendoza
Asunto: Submission Confirmation for your paper

Manuscript Title: DIARRHEA BY ROTAVIRUS IN A REGIONAL PERUVIAN HOSPITAL: DETERMINATION OF CIRCULATING GENOTYPES.
Corresponding Author: Dr Juana Mercedes del Valle Mendoza
Transactions of the Royal Society of Tropical Medicine and Hygiene

Dear Dr del Valle Mendoza,

Your submission entitled "DIARRHEA BY ROTAVIRUS IN A REGIONAL PERUVIAN HOSPITAL: DETERMINATION OF CIRCULATING GENOTYPES." has been received by journal Transactions of the Royal Society of Tropical Medicine and Hygiene

Your manuscript will be given a reference number once an Editor has been assigned.

You will be able to check on the progress of your paper by logging on as an author. The URL is <http://trstmh.edmgr.com/>.

Thank you for submitting your paper to this journal.

Kind regards,

Transactions of the Royal Society of Tropical Medicine and Hygiene

ESTADO DE PUBLICACIÓN

El 16 de Enero del año 2014, el artículo fue enviado a la revista *Transactions of the Royal Society of Tropical Medicine and Hygiene* para publicación. El 03 de Febrero del 2014 se recibieron las sugerencias y observaciones de la primera versión del artículo por parte de los editores de la revista. El 03 de Marzo del 2014 se envió la segunda versión corregida del manuscrito para publicación.

El autor principal se compromete a corregir todas las observaciones planteadas por la revista y, en caso sea rechazado, a enviar el artículo a otra revista indizada en SCOPUS.

COMPROMISO DE PUBLICACIÓN

Monterrico 20 de Enero del 2014

Sr.(a) Decano de la Facultad de Ciencias de la Salud


Por medio de la presente nos dirigimos a Ud. a fin de comunicarle nuestro interés por publicar, en la revista “Transactions of the Royal Society of Tropical Medicine and Hygiene”, el artículo titulado:

**DIARRHEA BY ROTAVIRUS IN A REGIONAL PERUVIAN HOSPITAL:
DETERMINATION OF CIRCULATING GENOTYPES.**

El cual es tema de tesis del autor principal: “Pablo Renato Weilg Espejo”, y fue supervisado por la asesora: Juana del Valle Mendoza

En este sentido, el autor se compromete a realizar el seguimiento respectivo con la finalidad de publicar el artículo en la revista mencionada.

Atentamente,



Pablo Weilg Espejo



PhD. Juana Del Valle Mendoza