Molecular epidemiology of noroviruses detected in Vietnamese children with acute gastroenteritis from 2012 to 2015

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Abstract
Noroviruses, an important cause of diarrhoea in humans, are genetically diverse. The recent norovirus seasons recorded the emergence of new recombinants of the capsid and polymerase genotypes, with a global dominance of GII.Pe_GII.4 Sydney 2012 and GII.P17_GII.17 in Asian countries. However, the number of papers reporting the distribution of both polymerase and capsid genotypes circulating among children is scarce, with none from Vietnam. This study described both the polymerase and capsid genotypes of noroviruses circulating in Vietnamese children using stool specimens obtained under the World Health Organization rotavirus surveillance programme from 2012 to 2015. Of 350 specimens tested, noroviruses were detected in 90 (28 %) of 319 inpatient specimens and in 9 (29 %) of 31 outpatient specimens. The polymerase and capsid genotype combinations of GII.Pe_GII.4 Sydney 2012 and GII.P21_GII.3 were co-dominant (51 and 24 %, respectively), both of which were recombinants, contributing to a high proportion (87 %) of recombinants among circulating noroviruses. GII.4 variants evolved in the same fashion in Vietnam as in other countries, with amino acid substitutions in the putative variant-specific epitopes of the protruding domain. Unlike neighbouring countries where the predominance of GII.P17_GII.17 was reported, only one GII.P17_GII.17 strain was detected from an outpatient in 2015 in Vietnam. In conclusion, a substantial burden due to norovirus gastroenteritis hospitalizations among Vietnamese children was associated with circulating co-dominant GII.Pe_GII.4 Sydney 2012 and GII.P21_GII.3 strains. Continued surveillance is necessary to monitor infection caused by GII.4 variants and that of GII.P17_GII.17 noroviruses in paediatric patients in Vietnam.

INTRODUCTION
Noroviruses are the second most important cause of acute gastroenteritis in children [1]. In countries where rotavirus vaccines are incorporated in the infant immunization schedule, a decline has been observed in hospitalizations due to rotavirus gastroenteritis with noroviruses becoming the main cause of severe acute gastroenteritis in children aged less than 5 years [2–5]. The norovirus genome comprises a single-stranded, positive-sense RNA possessing three ORFs, ORF1, ORF2 and ORF3, which respectively encode non-structural viral proteins including the RNA-dependent RNA polymerase (RdRp), the major capsid protein (VP1) and the minor capsid protein (VP2). Structurally, each VP1 monomer is divided into the shell domain (S) and the protruding domain (P), which is further divided into the P1 sub-domain and the P2 sub-domain. The P2 sub-domain reveals the putative variant-specific epitopes that are recognized by the generated human mAbs (blockade epitopes) [6, 7] and the putative histo-blood group antigen (HBGA) binding sites, which are the presumptive initial binding site in establishing human infection [8, 9].

The two main driving forces of norovirus genome evolution are accumulation of point mutations and recombination occurring between the ORF1 and ORF2 [10]. Noroviruses are classified into six genogroups (I–VI) based on the phylogenetic clustering of the complete VP1 amino acid sequence. Among these, genogroups I and II frequently infect humans [1]. The genogroup is further divided into genotypes and variants; genotypes are defined based on either the VP1 amino acid sequence divergence (the capsid genotype) or the nucleotide sequence divergence of the RdRp (the polymerase genotype). There are 9 and 22 capsid genotypes reported for GI and GII, respectively, and 14 and 26 polymerase genotypes reported for GI and GII, respectively [11].
Although noroviruses are genetically and antigenically diverse, a single capsid genotype 4 of genogroup II (GII.4) has been dominant for the past two decades, causing both sporadic cases and outbreaks of norovirus gastroenteritis [12–14]. The second most common genotype detected among children with norovirus gastroenteritis is GII.3 [13, 14]. It is therefore crucial that, if norovirus vaccine is to be developed, a formulation that protects against the capsid genotypes GII.4 and GII.3 should be prioritized. Indeed, a norovirus vaccine against GII.4 has been under clinical development [15].

On the other hand, minor genotypes have emerged in parts of the world. These include GII.12 in USA, Brazil, South Africa and China between 2007 and 2011, GII.13 in India and Nepal between 2009 and 2010 and in China in 2011 [16–22]. Also emerging in Japan was GII.14 between 2011 and 2013 as well as GII.21 in India in 2005 and in Bhutan between 2011 and 2012 [23, 24].

More recently, during the 2014–2015 epidemic season, there was an increase in the number of reports describing a sudden dominance of a minor capsid genotype GII.17 in Japan, China, Taiwan and Hong Kong, apparently replacing the almost-two-decade-dominant GII.4 genotype [25–31]. The emergence and increase of the non-GII.4 genotypes may be a challenge against the candidate norovirus vaccines [15], which target the globally predominant GII.4 noroviruses, as it is not known whether they are cross-protective against non-GII.4 noroviruses.

While the major capsid protein is the primary target of the protective antibodies, recombination resulting in the acquisition of new polymerase genotypes is considered another mechanism of gaining the capacity to establish an efficient human-to-human spread [32]. Thus, it is desirable to characterize both capsid and polymerase genotypes in routine surveillance to better understand the molecular epidemiology of noroviruses, including the prevalence of recombinant strains [33].

However, papers reporting the distribution of both polymerase and capsid genotypes of noroviruses circulating among children are scarce, and none has so far been reported from Vietnam (Table S1, available in the online Supplementary Material). We, therefore, conducted this study to obtain insight into the circulating polymerase and capsid genotypes of noroviruses in children less than 5 years of age who were hospitalized for acute gastroenteritis in Vietnam, intending to understand the extent of genetic recombination in noroviruses circulating in the children in Vietnam.

METHODS

As part of an ongoing World Health Organization rotavirus surveillance programme in Vietnam, a total of 6259 specimens were collected from children less than 5 years of age hospitalized for diarrhea from 2012 to 2015 at three hospitals, namely, the National Hospital of Paediatrics located in Hanoi (north), Ninh Hoa General Hospital and Khanh Hoa General Hospital located in Khanh Hoa (centre). Of these, 350 specimens were selected by systematic random sampling using a norovirus-associated diarrhoea hospitalization rate of 28 % [34] and a lower limit of 95 % confidence interval.

The genomic RNAs were extracted from stool suspensions by using a QIAamp Viral RNA Mini kit (Qiagen); cDNA was synthesized from the extracted RNA by using random hexamers and SuperScript III First-Strand Synthesis System (Invitrogen). Norovirus genogroups I and II were detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using the primers targeting the conserved ORF1–ORF2 junction region (region C) and G0f9q Green Master Mix (Promega). The norovirus capsid genotypes were determined by sequencing the amplicon of region C using Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems). The polymerase genotypes were determined by amplifying and sequencing the continuous sequence of about 800 nt at the 3’ end of ORF1 (RdRp sequence) and 300 nt of the N/S region. Representative samples of the GII.4 and GII.17 genotypes were amplified and sequenced for the entire capsid gene (ORF2).

The genotypes were determined using the Norovirus Automated Genotyping Tool online (http://www.rivm.nl/mpf/norovirus/typingtool/). Genetic and phylogenetic analyses were conducted using MEGA v7.0.14 [35]. Recombination events were identified by SimPlot v. 3.5.1 [36]. Homology modelling was performed for GII.4 Sydney 2012 strains using a fully automated protein structure homology-modelling server, SWISS-MODEL (http://swissmodel.expasy.org/), with the templates of 2obr and 4op7 [37]. Visualization and superposition were performed on NOC 3.01 (noch.sourceforge.net/).

RESULTS

Detection of noroviruses

Out of 350 specimens randomly selected from 6259 specimens, 319 were collected from hospitalized children (i.e. inpatients) of which 90 (28.2 %) were positive for norovirus with yearly variation from 23.4 to 33.0 % (Table 1). Only in 2015, samples from outpatients were examined for norovirus and 9 (29.0 %) tested positive. There was no difference in the detection rates between inpatients and outpatients (P<0.46).

Distribution of the capsid and polymerase genotypes of noroviruses

All of the 99 noroviruses detected in this study belonged to genogroup II of which 85 (86 %) were genotyped for the VP1 capsid (Table 1); GII.4 and GII.3 were the most and second most dominant throughout the study period and accounted for 55 and 22 %, respectively. These genotypes were followed by three GII.6 and two GII.17; one GII.17 was detected from an inpatient in 2012, whereas the other from an outpatient in 2015.
Table 2 shows the distribution of the 67 specimens for which both the capsid and polymerase genotypes were determined. Notably, 58 (87 %) possessed discordant polymerase and capsid genotypes; hence, they were inter-genotype recombinants. Specifically, GII.Pe_GII.4 Sydney 2012 (n=34) and GII.P21_GII.3 (n=16) accounted for the majority of the recombinants. GII.P17_GII.17 detected in 2015 was also determined to be recombinant as previously circulating GII.17 strains, which were isolated worldwide by 2012, were found to be accompanied by other polymerase genotypes, i.e. GII.P4, GII.P13 or GII.P16 [38], and the capsid genotype GII.17 was suggested to evolve by exchanging the RdRp gene through the recombination events [26].

When the distribution of GII.4 variants over the study period was examined, GII.P4 Den Haag 2006b_GII.4 Den Haag 2006b (GII.4 Den Haag 2006b) prevailed in 2012 and was last seen in the following year (Fig. 1). GII.4 Den Haag 2006b was replaced by GII.Pe_GII.4 Sydney 2012 (GII.4 Sydney 2012). The most predominant GII.4 variant during the surveillance period was GII.4 Sydney 2012, appearing for the first time in 2012 and continuing to prevail until 2015 (Fig. 1).

Genetic characteristics of the VP1 gene of Vietnamese GII.4 variants
Nine strains detected at different times of the study period were selected as representatives of four of the different combinations of the polymerase and capsid genotypes of the GII.4 variants: GII.P4 Den Haag 2006b_GII.4 Den Haag 2006b, GII.P4 New Orleans 2009_GII.4 New Orleans 2009, GII.P4 New Orleans 2009_GII.4 Sydney 2012 and GII.Pe_GII.4 Sydney 2012. They were sequenced for the entire capsid and partial polymerase genes. When a subtree of the VP1 gene tree that contained the Vietnamese GII.P4 Den Haag 2006b_GII.4 Den Haag 2006b sequence (Hu/GII.4/
NVN12.214/VNM/2012) and other GII.4 Den Haag 2006b variant sequences retrieved from the GenBank database was examined (Fig. 2a), Hu/GII.4/NVN12.214/VNM/2012 was the closest to Vietnamese GII.4 Den Haag 2006b strains detected in 2011 and 2009 with nucleotide sequence identities ranging from 98.3 to 99.1 %, than to globally circulating GII.4 Den Haag 2006b variants with identities ranging from 97.0 to 97.7 %.

When another subtree of the VP1 gene tree that contained the GII.4 Sydney 2012 variants detected in Vietnam and elsewhere in the world was examined (Fig. 2b), the Vietnamese strains clustered into three separate lineages/sublineages: the GII.Pe_GII.4 Sydney 2012 sub-lineage consisting of strains detected in 2013 (indicated with squares in Fig. 2b), the GII.Pe_GII.4 Sydney 2012 lineage consisting of strains detected in 2014 and 2015 (indicated with dots) and the GII.P4 New Orleans 2009_GII.4 Sydney 2012 lineage consisting of strains detected in 2015 (indicated with diamonds). First, two Vietnamese GII.Pe_GII.4 Sydney 2012 strains detected in 2013 were the closest to globally circulating GII.4 Sydney 2012 variants detected in 2012 with nucleotide sequence identities of more than 99 %. Second, four Vietnamese GII.Pe_GII.4 Sydney 2012 strains detected in 2014 and 2015 were the closest to the strains detected in Japan, China and Hong Kong circulating in 2014 with nucleotide sequence identities ranging from 98.3 to 99.4 %. Third, the Vietnamese GII.P4 New Orleans 2009_GII.4 Sydney 2012 strain detected in 2015 was the closest to two strains detected in Hong Kong in 2015 and Japan in 2012 with a mean nucleotide sequence identity of 98.4 % (Fig. 2b).

**Structural characteristics of the VP1 protein of Vietnamese GII.4 variants**

Based on analysis of amino acid variations in the P2 domain of different strains of Vietnamese GII.4 Sydney 2012 variants, it was found that variations occurred in the putative variant-specific epitopes (blockade epitopes) A, B, C and D, but no variation occurred in epitope E (Fig. 3a). Amino acid substitutions occurring at residues adjacent to the antigen-icity-determining sites and HBGA-binding sites were observed (Fig. 3a), and such substitutions resulted in obvious or slight changes in the structure (Fig. 3b) and the electrostatic surface potential of the P dimers.

Specifically, analyses of the P2 domain of two GII.Pe_GII.4 Sydney 2012 strains, Hu/GII.4/NVN13.400/VNM/2013 which was detected in April, 2013, and Hu/GII.4/NVN13.1441/VNM/2013 detected 6 months later, revealed the substitutions I293T, R297H and R373H that resulted in structural changes of the loop (residues 294–298) at the blockade epitope A, transforming this epitope from positively charged to negatively charged. The substitution T340I at the blockade epitope C was also found.

Analyses of the P2 domain of the GII.4 Sydney 2012 variants detected in 2013 and the ones detected during 2014 and 2015 revealed amino acid substitution V333M in the blockade epitope B. The GII.4 Sydney 2012 variants detected in 2014 and 2015 carried either R373 or H373 (Fig. 3a) that resulted in a slight change in the electrostatic surface potential of the P dimers. Similarly, substitutions G393 and H414 found in the P2 region of the GII.P4 New Orleans 2009_GII.4 Sydney 2012 strain, Hu/GII.4/
NVN15.706/VNM/2015, caused a structural variation in the blockade epitopes D and E (Fig. 3b).

**Genetic characteristics of the VP1 of the Vietnamese GII.P17_GII.17 strain**

When a phylogenetic tree was drawn for the VP1 genes of Hu/GII.P17_GII.17/NVN15.349/VNM/2015 together with the GII.17 strains available in the GenBank, the Vietnamese strain belonged to the sub-cluster of Kawasaki_308/2014-like strains that caused outbreaks in Asian countries including China, Taiwan and Hong Kong between 2014 and 2015 with nucleotide sequence identities ranging from 98.7 to 99.8 % within the cluster (Fig. 4a). Phylogenetic analysis on the sequences of about 750 nt at the 3′ end of the RdRp gene for Hu/GII.P17_GII.17/NVN15.349/VNM/2015 and other GII.17 strains revealed that the Vietnamese strain was closely related to the ones of which the VP1 belonged to the sub-cluster of Kawasaki_308/2014-like strains with nucleotide sequence identities ranging from 99 to 99.6 % (Fig. 4b).

**DISCUSSION**

In this study, norovirus was detected in 28.2 % of Vietnamese children less than 5 years of age who were hospitalized for acute gastroenteritis from 2012 to 2015. This detection rate was in good agreement with the rates reported in earlier studies in Vietnam [34, 39] but higher than the global
average (17%, CI 15–19) [40]. When the detection rate of 28% was taken together with the incidence rate of diarrhoea hospitalizations (60.8/1000 child years) reported in central Vietnam [41], the annual incidence rate of norovirus hospitalization was calculated to be 17 per 1000 child years among children less than 5 years of age, suggesting the presence of a high disease burden due to norovirus in Vietnam.

In this study, the detection rates of norovirus in both inpatient and outpatient groups were shown to be similar, which is consistent with results previously reported elsewhere in the world [21, 42, 43]. This observation makes a strong contrast with...
The putative variant-specific epitope of GII.4
[6, 7, 61]

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The putatively common HBGA-binding sites [8,9]

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Newly identified HBGA-binding sites [9]

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Amino acid position on the VP1

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GII/Ho/VNM/2015/GII.4/ NVN15.496 T . . . . . . N M . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GII/Ho/VNM/2015/GII.4/ NVN15.506 T . . . . . . N M . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GII/Ho/VNM/2015/GII.4/ NVN15.706 T . . . . . . N M . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

*Positions were under significant positive selection [61].**HBGA-binding site 1 was formed by loops of amino acids interacting with α-fucose ring; HBGA-binding site 2 consists of amino acids interacting with α-N-acetylglucosamine, β-galactose or Lewis-fucose ring [8,9].

Fig. 3. Amino acid variation in the P2 region of GII.4 Sydney strains detected in Vietnam during 2013–2015. (a) Amino acid substitutions in the P2 region. (b) Top view of superposition of the models of the P dimers of GII.4 Sydney strains and location of functional loops. *, Positions were under significant positive selection [61].
what was found in rotavirus-infected children in which the relative frequency among the inpatient group was twice as high as that among the outpatient group [44–47].

While this study confirmed the co-dominance of the GII.4 and GIIL3 genotypes in Vietnam similar to earlier studies [39, 48–53], it was found that these dominant GII.4 and GIIL3 capsid genotypes possessed discordant polymerase genotypes GII.Pe and GII.P21, respectively, leading to a high proportion (87 %) of recombinant noroviruses. The recombinant genotype GII.P21_GII.3 has been reported to be the second most dominant genotype, next to GII.Pe, each combination represents intra-genotype or inter-genotype recombinant norovirus, respectively [18, 54].

The proportion of recombinants was not invariant over the study period, but it abruptly increased from 42 % in 2012 to more than 95 % thereafter (Table 2). While the recombinant strains in 2012 comprised GII.3 strains (i.e., GII.P12_GII.3 and GII.P21_GII.3), GII.Pe_GII.4 Sydney 2012 constituted the vast majority of the recombinants between 2013 and 2015. The abrupt increase in the recombinants due to the predominance of GII.Pe_GII.4 Sydney 2012 was also reported from China (31–56 % in 2010–2012 to 100 % in 2013) and South Africa (29–42 % in 2009–2012 to 84 % in 2013) [18, 54]. The high proportion of recombinants was also associated with circulation of other recombinant noroviruses, including GII.P7_GII.6, GII.P16_GII.13 and GII.P17_GII.17, which were reported to be recently emerging genotypes elsewhere in the world [18, 21, 27, 29, 54].

**Fig. 4.** Phylogenetic analyses on the partial RdRp and complete VP1 genes of a GII.17 strain detected in Vietnam and those detected elsewhere in the world. Phylogenetic analysis was conducted in MEGA7 software package using the maximum-likelihood method based on the Kimura 2-parameter model by assuming that a certain fraction of sites are evolutionarily invariable (+I) for the RdRp sequences and the Hasegawa–Kishino–Yano model with a discrete gamma distribution for the VP1 genes. Bootstrap values were obtained after 1000 bootstrap replicates, and values >80 % are shown. The trees are drawn to scale, with branch lengths measured in number of substitutions per site. The outgroup strain (Hu/GII/9/Goulburn Valley G5175 C/USA/1983) was not shown in the phylogenetic tree of the RdRp gene (about 750 nt) possessed by GII.17 norovirus strains.
observations led us to hypothesize that acquiring a new polymerase genotype in addition to a new capsid genotype may be a requirement for any new norovirus strain to emerge and spread in the population. Thus, the importance of genotyping not only capsid but also polymerase genotypes in norovirus surveillance needs to be recognized [11, 33].

In this study, in 2012, GII.4 Den Haag 2006 was the predominant variant of GII.4 with two other GII.4 variants, GII.4 New Orleans 2009 and GII.4 Sydney 2012, co-circulating, but the GII.4 Sydney 2012 strain became predominant thereafter. The finding was consistent with previous findings in Vietnam. Specifically, in Vietnam, GII.4 Den Haag 2006 was reported to prevail between 2007 and 2012, although there was occurrence of other variants such as GII.4 New Orleans 2009 since 2010 and GII.4 Sydney 2012 since 2012 [48, 49, 57]. At the global level, GII.4 Den Haag 2006 was reported to be the predominant variant of GII.4 worldwide since 2006 [13]. This variant was successively replaced by other GII.4 variants including GII.4 New Orleans 2009 since 2009–2010 and GII.4 Sydney 2012 since 2012 [23, 52, 54, 56, 58, 59], although the dominance of GII.4 Den Haag 2006 was still reported until 2011–2012 in a few countries including China [51, 54, 60].

Phylogenetic analysis revealed that the sources from which GII.4 strains were introduced in the population might be variable. Our analysis suggested that the origin of the Vietnamese GII.4 Den Haag 2006 strain detected in 2012 was the local strains previously circulating in the country, while the origins of Vietnamese GII.4 2012 strains were different.

The accumulation of point mutations in the P2 domain, particularly those within the functional loops of GII.4 Sydney 2012 strains, was shown to cause the emergence of new variants in the population [6, 8]. Among five blockade epitopes A–E, the putative variant-specific epitopes A, D and E are large and important ones of GII.4 variants [6]. Mutations on the residues of epitopes A and D among GII.4 Sydney 2012 strains prevailing worldwide were considered to change the antigenicity of the viruses and to cause the emergence of the viruses in the population [7, 61]. Mutations in or around the HBGA-binding sites that results in negative charge of the P dimers may play a role in expanding the HBGA-binding spectrum of recently circulating GII.4 strains because the regions are mostly negatively charged [9]. Therefore, for Vietnamese GII.4 Sydney 2012 strains, the mutations having occurred in epitope A, the most important neutralization escape determinant, and in epitope D, another important blockade epitope [6], may cause antigenic variations for GII.4 Sydney 2012 strains in different genetic lineages to overcome the herd protection against the preceding norovirus variants. In addition, the mutations occurring at and around the loops of HBGA-binding sites result in structural changes of the HBGA-binding pockets, which are believed to alter HBGA-binding profiles of GII.4 noroviruses [8]. Thus, it is likely that the GII.4 Sydney 2012 strains may adapt to new populations or expand the range of susceptible populations. Another mechanism may be that capsid genotype GII.4 Sydney 2012 was combined with a new polymerase genotype GII.Pc, creating an evolutionary advantage in producing a diverse pool of progeny viruses from which fitter variants may be selected.

Recently, there have been reports of new GII.P17_GII.17 strains as a major cause of norovirus outbreaks in Japan, China, Taiwan and Hong Kong in 2014 and 2015 as well as sporadic cases in China and Hong Kong [22, 25–29, 31]. In our study, however, only one GII.P17_GII.17 strain was detected in an outpatient despite the fact that the Vietnamese GII.P17_GII.17 strain belonged to the same sub-cluster as the prevailing GII.P17_GII.17 strains, possessing the profile of HBGA-binding sites identical with the ones of outbreak-causing strains in China and Hong Kong [29].

Previous reports showed that the infection with the emergent GII.P17_GII.17 was more likely to occur among older children and adults [27, 29–31, 62, 63]. Another report from a study conducted among Italian children hospitalized with acute gastroenteritis between September 2014 and March 2015 showed that GII.17 accounted for only 2 (2.5%) of 81 norovirus-positive samples [63]. Thus, the emerging GII.P17_GII.17 strains may not be as capable of spreading among infants and young children as they are in older children and adults.

In conclusion, this study showed that norovirus imposed a substantial burden on Vietnamese children aged less than 5 years, with GII.Pc_GII.4 Sydney 2012 variants and GII.P21_GII.3 being co-dominant. GII.4 variants evolved in the same fashion in Vietnam as elsewhere in the world, with amino acid substitutions in the major epitopes of the P domain. Unlike neighbouring countries where emerging GII.P17_GII.17 noroviruses caused outbreaks during the 2014–2015 season, only a single GII.P17_GII.17 strain was detected from an outpatient in 2015. Continued surveillance is necessary to monitor infection caused by GII.4 variants and that of GII.P17_GII.17 noroviruses in paediatric patients in Vietnam.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study did not conduct any experimental work with humans. The use of stool specimens in the study was reviewed and approved by the institutional review board.
the Institutional Review Board of the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, with an ethical approval number of IRB-VN01057-24/2015.

References


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