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Introduction to Agarose and Polyacrylamide Gel Electrophoresis Matrices with Respect to Their Detection Sensitivities

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1. Introduction

During the last years molecular biology techniques, such as polymerase chain reaction (PCR), have become widely used for medical and forensic applications, as well as research, and detection and characterization of infectious organisms. In the virology field, it has been demonstrated that the employment of PCR technique offers the advantages of high sensitivity and reproducibility in viral genomic detection and strains characterization. However, the sensitivity in the detection of DNA fragments is also linked to the sensitivity of the electrophoresis matrix applied for PCR product development.

Electrophoresis through agarose or polyacrylamide gels is a standard method used to separate, identify and purify nucleic acids, since both these gels are porous in nature. In this chapter the evaluation of the sensitivity of agarose and polyacrylamide gel electrophoresis matrices in the detection of PCR products is analyzed. For this purpose, rotavirus PCR amplicons were used as a model.

Human rotaviruses have been recognized as the most common cause of dehydrating diarrhea in infants and young children on worldwide scale. These viruses are characterized by the presence of 11 segments of double-stranded RNA surrounded by three separate shells, the core, inner capsid and outer capsid. Currently, rotaviruses are dual classified into G and P genotypes according to the differences of VP7 and VP4 neutralization antigens which form the outer capsid of the virion. Two rotavirus vaccines have been licensed in the year 2006 in many countries. Although large-scale safety and efficacy studies of both rotavirus vaccines have shown excellent efficacy against severe rotavirus gastroenteritis (Ruiz-Palacios et al., 2006; Matson, 2006), the lack of clear data about the protection against genotypes not included in the vaccine formulations underlines the importance of virological surveillance, rotavirus strain characterization and the evaluation of the impact of these vaccines in diminishing the diarrhea illness in our region (Gentsch et al., 2005; Perez-Schael et al., 1990; Velazquez et al., 1996).

In addition, the presence of multiple G and/or P genotypes in individual specimens may offer an unique environment for mixed infection acquisition and thereby for the

reassortment of rotavirus genes. This could affect both, rotavirus evolution and efficacy performance of current and future vaccines. In this context, knowledge of both the rotavirus genotypes circulating in a community and the incidence of rotavirus mixed infections is essential for acquiring an in-depth understanding of the ecology and distribution of rotavirus strains and anticipating antigenic changes that could affect vaccine effectiveness.

For this purpose, rotavirus G and P genotypes are determined by extraction of the viral RNA from fecal specimens followed by analysis by semi-nested reverse-transcriptase PCR (RT-PCR) with primers specific for regions of the genes encoding the VP7 or VP4. The genotype-specific PCR products are then analyzed on an agarose or polyacrylamide gel followed by ethidium bromide staining or silver staining, respectively.

The matrix used for electrophoresis should have adjustable but regular pore sizes and be chemically inert, and the choice of which gel matrix to use depends primarily on the sizes of the fragments being separated (Guilliatt, 2002). As commented before, although the importance of specificity and sensitivity of PCR is well known, the mechanism by which the results are measured is equally important (Wildt et al., 2008).

2. General characteristics of agarose and polyacrylamide matrices

2.1 Agarose gel electrophoresis (AGE)

Agarose is a natural linear polymer extracted from seaweed that forms a gel matrix by hydrogen-bonding when heated in a buffer and allowed to cool. For most applications, only a single-component agarose is needed and no polymerization catalysts are required. Therefore, agarose gels are simple and rapid to prepare (Chawla, 2004). They are the most popular medium for the separation of moderate and large-sized nucleic acids and have a wide range of separation but a relatively low resolving power, since the bands formed in the gels tend to be fuzzy and spread apart. This is a result of pore size and cannot be largely controlled. These and other advantages and disadvantages of using agarose gels for DNA electrophoresis are summarized in Table 1 (Stellwagen, 1998).

Advantages	Disadvantages
Nontoxic gel medium	High cost of agarose
Gels are quick and easy to cast	Fuzzy bands
Good for separating large DNA molecules	Poor separation of low molecular weight samples
Can recover samples by melting the gel, digesting with enzyme agarose or treating with chaotropic salts	

Table 1. Advantages and disadvantages of agarose gel electrophoresis.

2.2 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels are chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually N,N'-methylenebisacrylamide. The reaction is a free radical polymerization, usually carried out with ammonium persulfate as the initiator and N,N,N',N'-tetramethylethylendiamine (TEMED) as the catalyst. Although the gels are generally more difficult to prepare and handle, involving a longer time for preparation than agarose gels, they have major advantages over agarose gels. They have a

greater resolving power, can accommodate larger quantities of DNA without significant loss in resolution and the DNA recovered from polyacrylamide gels is extremely pure (Guilliatt, 2002). Moreover, the pore size of the polyacrylamide gels can be altered in an easy and controllable fashion by changing the concentrations of the two monomers. Anyway, it should be noted that polyacrylamide is a neurotoxin (when unpolymerized), but with proper laboratory care it is no more dangerous than various commonly used chemicals (Budowle & Allen, 1991). Some advantages and disadvantages of using polyacrylamide gels for DNA electrophoresis are depicted in Table 2 (Stellwagen, 1998).

Advantages	Disadvantages
Stable chemically cross-linked gel	Toxic monomers
Sharp bands	Gels are tedious to prepare and often leak
Good for separation of low molecular weight fragments	Need new gel for each experiment
Stable chemically cross-linked gel	

Table 2. Advantages and disadvantages of polyacrylamide gel electrophoresis.

3.Gel concentration

3.1 Agarose gel concentration

The percentage of agarose used depends on the size of fragments to be resolved. The concentration of agarose is referred to as a percentage of agarose to volume of buffer (w/v), and agarose gels are normally in the range of 0.2% to 3% (Smith, 1993). The lower the concentration of agarose, the faster the DNA fragments migrate. In general, if the aim is to separate large DNA fragments, a low concentration of agarose should be used, and if the aim is to separate small DNA fragments, a high concentration of agarose is recommended (Table 3).

Concentration of agarose (%)	DNA size range (bp)
0.2	5000-40000
0.4	5000-30000
0.6	3000-10000
0.8	1000-7000
1	500-5000
1.5	300-3000
2	200-1500
3	100-1000

Table 3. Agarose gel concentration for resolving linear DNA molecules.

3.2 Polyacrylamide gel concentration

The choice of acrylamide concentration is critical for optimal separation of the molecules (Hames, 1998). Choosing an appropriate concentration of acrylamide and the cross-linking agent, methylenebisacrylamide, the pore sized in the gel can be controlled. With increasing the total percentage concentration (T) of monomer (acrylamide plus cross-linker) in the gel,

the pore size decreases in a nearly linear relationship. Higher percentage gels (higher T), with smaller pores, are used to separate smaller molecules. The relationship of the percentage of the total monomer represented by the cross-linker (C) is more complex. Researchers have settled on C values of 5% (19:1 acrylamide/bisacrylamide) for most forms of denaturing DNA and RNA electrophoresis, and 3.3% (29:1) for most proteins, native DNA and RNA gels. For optimization, 5% to 10% polyacrylamide gels with variable cross-linking from 1% to 5% can be used. Low cross-linking (below 3% C) yields "long fiber gels" with increased pore size (Glavač & Dean, 1996). Moreover, it should be pointed out that at low acrylamide/bisacrylamide concentrations the handling of the gels is difficult because they are slimy and thin. Table 4 gives recommended acrylamide/bisacrylamide ratios and gel percentages for different molecular size ranges.

Acrylamide/Bis Ratio	Gel %	Native DNA/RNA (bp)	Denatured DNA/RNA (bp)
19:1	4	100-1500	**70-500**
	6	60-600	**40-400**
	8	40-500	**20-200**
	10	30-300	**15-150**
	12	20-150	**10-100**
29:1	5	**200-2000**	70-800
	6	**80-800**	50-500
	8	**60-400**	30-300
	10	**50-300**	20-200
	12	**40-200**	15-150
	20	**<40**	<40

Table 4. Polyacrylamide gel concentration for resolving DNA/RNA molecules. Note: Recommended applications for each formulation are shown in **bold**.

4. Electrophoretic buffer systems

Effective separation of nucleic acids by agarose or polyacrylamide gel electrophoresis depends upon the effective maintenance of pH within the matrix. Therefore, buffers are an integral part of any electrophoresis technique. Moreover, the electrophoretic mobility of DNA is affected by the composition and ionic strength (salt content) of the electrophoresis buffer (Somma & Querci, 2006). Without salt, electrical conductance is minimal and DNA barely moves. In a buffer of high ionic strength, electrical conductance is very efficient and a significant amount of heat is generated. Different categories of buffer systems are available for electrophoresis: dissociating and non-dissociating, continuous and discontinuous.

4.1 Dissociating and non-dissociating buffer systems

The electrophoretic analysis of single stranded nucleic acids is complicated by the secondary structures assumed by these molecules. Separation on the basis of molecular weight requires the inclusion of denaturing agents, which unfold the DNA or RNA strands and remove the influence of shape on their mobility. Nucleic acids form structures stabilized by hydrogen bonds between bases. Denaturing requires disrupting these hydrogen bonds. The most

commonly *dissociating buffer systems* used include urea and formamide as DNA denaturants. Denatured DNA migrates through these gels at a rate that is almost completely dependent on its base composition and sequence. Denaturing or dissociating buffer systems for proteins include the use of sodium dodecyl sulfate (SDS). In the SDS-PAGE system, developed by Laemmli (1970), proteins are heated with SDS before electrophoresis so that the charge-density of all proteins is made roughly equal. Heating in SDS, an anionic detergent, denatures proteins in the samples and binds tightly to the uncoiled molecule (with net negative charge). Consequently, when these samples are electrophoresed, proteins separate according to mass alone, with very little effect from compositional differences. DNA molecules are negatively charged; therefore the addition of SDS in the gel preparations is only with the aim of enhancing the resolution power of the bands (Day & Humphries, 1994).

In the absence of denaturants, double stranded DNA (dsDNA), like a PCR product, retains its double helical structure, which gives it a rodlike form as it migrates through a gel. During the electrophoresis of native molecules in a *non-dissociating buffer system*, separation takes place at a rate approximately inversely proportion to the $\log_{10}$ of their size.

4.2 Continuous and discontinuous buffer systems

In the *continuous buffer systems* the identity and concentration of the buffer components are the same in both the gel and the tank. Although continuous buffer systems are easy to prepare and give adequate resolution for some applications, bands tend to be broader and resolution consequently poorer in these gels. These buffer systems are used for most forms of DNA agarose gel electrophoresis, which commonly contain EDTA (pH 8.0) and Tris-acetate (TAE) or Tris-borate (TBE) at a concentration of approximately 50mM (pH 7.5-7.8). TAE is less expensive, but not as stable as TBE. In addition, TAE gives better resolution of DNA bands in short electrophoretic separations and is often used when subsequent DNA isolation is desired. TBE is used for polyacrylamide gel electrophoresis of smaller molecular weight DNA (MW<2000) and agarose gel electrophoresis of longer DNA where high resolution is not essential.

Discontinuous (multiphasic) systems employ different buffers for tank and gel, and often two different buffers within the gel. Discontinuous systems concentrate or "stack" the samples into a very narrow zone prior to separation, which results in improved band sharpness and resolution. The gel is divided into an upper "stacking" gel of low percentage of acrylamide and low pH (6.8) and a separating gel with a pH of 8.8 and much smaller pores (higher percentage of acrylamide). The stacking gel prevents any high-molecular-weight DNA present in the sample from clogging the pores at the top of the running gel before low-molecular-weight DNA has entered. Both, the stacking and the separating gels, contain only chloride as the mobile anion, while the tank buffer contains glycine as its anion, at a pH of 8.8. The major advantage of the discontinuous buffer system over continuous buffer system is that this gel system can tolerate larger sample volumes (Rubin, 1975).

5. Loading buffer

This is the buffer to be added to the DNA fragment that will be electrophoresed. This buffer contains glycerol or sucrose to increase the density of the DNA solutions; otherwise, the samples would dissolve in running buffer tank and not sink into the gel pocket. The gel

loading buffer also contains dyes that facilitate observation of the sample during gel loading and electrophoresis, such as bromophenol blue or xylene cyanol. Because these molecules are small, they migrate quickly through the gel during electrophoresis, thus indicating the progress of electrophoresis (Chawla, 2004). The components and concentrations of the 6X loading dye usually used are: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol; or 0.25% bromophenol blue, 50 mM EDTA, 0.4% sucrose.

6. Voltage/current applied

The higher the voltage/current, the faster the DNA migrates. If the voltage is too high, band streaking, especially for DNA≥12-15kb, may result. Moreover, high voltage causes a tremendously increase in buffer temperature and current in very short time. The high amount of the heat and current built up in the process leads to the melting of the gel, DNA bands smiling, decrease of DNA bands resolution and fuse blowout. Therefore, it is highly recommended not exceed 5-8 V/cm and 75 mA for standard size gels or 100 mA for minigels. On the other side, when the voltage is too low, the mobility of small (≤1kb) DNA is reduced and band broadening will occur due to dispersion and diffusion.

7. Visualizing the DNA

After the electrophoresis has been completed there are different methods that may be used to make the separated DNA species in the gel visible to the human eye.

7.1 Ethidium bromide staining (EBS)

The localization of DNA within the agarose gel can be determined directly by staining with low concentrations of intercalating fluorescent ethidium bromide dye under ultraviolet light. The dye can be included in both, the running buffer tank and the gel, the gel alone, or the gel can be stained after DNA separation. For a permanent record, mostly instant photos are taken from the gels in a dark room. It is important to note that ethidium bromide is a potent mutagen and moderately toxic after an acute exposure. Therefore, it is highly recommended to handle it with considerable caution.

7.2 Silver staining (SS)

Silver staining is a highly sensitive method for the visualization of nucleic acid and protein bands after electrophoretic separation on polyacrylamide gels. Nucleic acids and proteins bind silver ions, which can be reduced to insoluble silver metal granules. Sufficient silver deposition is visible as a dark brown band on the gel. All silver staining protocols are made of the same basic steps, which are: i) fixation to get rid of interfering compounds, ii) silver impregnation with either a silver nitrate solution or a silver-ammonia complex solution, iii) rinses and development to build up the silver metal image, and iv) stop and rinse to end development prior to excessive background formation and to remove excess silver ion (Chevallet et al., 2006).

8. Objective of this study

The aim of the study presented in this chapter was to analyze the influence of the gel electrophoresis matrix (agarose and polyacrylamide) and staining system (ethidium

bromide and silver staining) in the detection of rotavirus G genotype amplicons (products of dsDNA).

9. Materials and methods

9.1 Rotavirus G genotype amplicon collection

A specimen collection of 2148 stool samples was obtained from children under 3 years of age who were hospitalized at different public and private hospitals in Córdoba City, Argentina, during the period 1979-2009. Out of the 2148 stool specimens, a total of 590 (27.5%) were positive for rotavirus infection and all of them were G genotype characterized by RT-PCR followed by heminested-PCR. Briefly, extracted RNA from the stool samples was reverse-transcribed into VP7-gene full length cDNA with the generic primers Beg9/End9. Then, the cDNA product was used as template for PCR VP7-amplification with the same Beg9/End9 pair of primers. The VP7 full length PCR products were used as templates in combination with two cocktails of type-specific forward primers and the generic reverse primer End9 for G-genotyping (Gouvea et al., 1990). The cocktails were as follows: G1 (aBT1), G2 (aCT2), and G3 (aET3) in one mixture, and G4 (aDT4), G8 (aAT8) and G9 (aFT9) in the second one. The amplicons obtained were comparatively analyzed by the standard agarose gel electrophoresis and ethidium bromide staining (AGE/EBS) method and polyacrylamide gel electrophoresis and silver staining (PAGE/SS). Those amplicons which showed discordant results were sequenced in order to verify the specificity of the visualized bands.

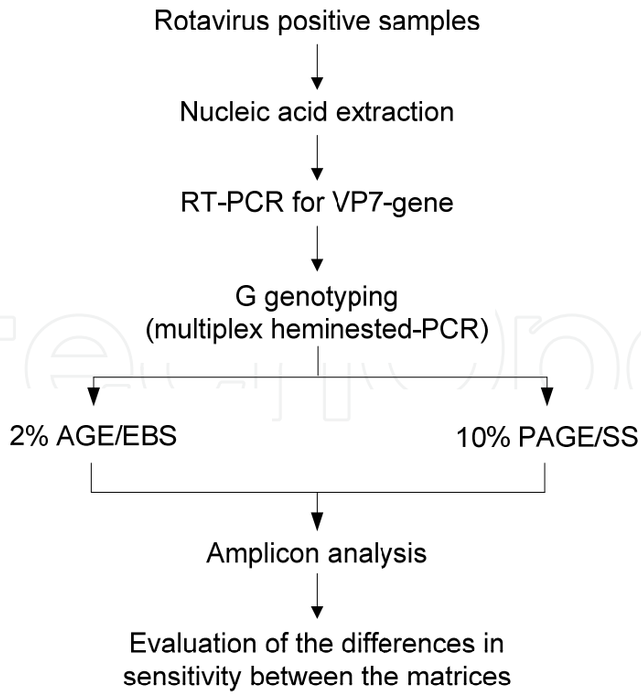

Fig. 1. Algorithm for the evaluation of the differences in sensitivity between agarose and polyacrylamide gel electrophoresis matrices in nucleic acid detection.

The algorithm carried out for the evaluation of the differences in sensitivity between agarose and polyacrylamide gel electrophoresis matrices in nucleic acid detection is shown in Figure 1.

9.2 Preparing, running and staining 2% agarose gels

The expected sizes of the genotype-specific PCR products were 749bp (G1), 652bp (G2), 374bp (G3), 583bp (G4), 885bp (G8), and 306bp (G9). Therefore, 2% agarose concentration was used for the electrophoresis of the PCR amplicons (Table 5). Agarose gels were treated with ethidium bromide for later visualization of DNA amplicons (final concentration 0.5 ug/ml). The ethidium bromide was added to the gel preparation in order to minimize ethidium bromide-containing waste. Equal volumes of 10ul of the heminested-PCR products and Phyndia buffer (0.02M Tris-HCl pH 7.4, 0.3M NaCl, 0.01M $MgCl_2$, 0.1% SDS, 5mM EDTA, 4% sucrose, 0.04% bromophenol blue) were mixed and load onto the gels, along with a 100pb DNA ladder, for later comparison of amplicon sizes. Agarose gels were electrophoresed in running buffer TBE (0.09M Tris-Borate, 0.002M EDTA) for 30-60min at 80-100V. After the run, PCR products were visualized in UV transilluminator.

Solution	Quantity/Volume
Agarose	2 gr
Ethidium bromide (10 mg/ml)	5 ul
Deionized water	100 ml

Table 5. Recipe for the preparation of 2% agarose gels.

9.3 Preparing, running and staining 10% polyacrylamide gels

As PCR expected amplicon sizes are in the range of 306-749bp, 6% polyacrylamide gels concentration should be used, as this concentration is recommended for the separation of products between 80 and 800bp. However, the handling of these gels was difficult as they were too slimy. For this reason, gel concentration was increased to a 10% in the separating gel, achieving good separation of all the PCR amplicons in gels of this concentration. Equal volumes of 10ul of the heminested-PCR products and Phyndia buffer were mixed and load onto 10% polyacrylamide gels of 1mm thickness. Along with the PCR products, a 100pb

Separating gel		Stacking gel	
Solution	**Volume**	**Solution**	**Volume**
Acrylamide 30%	2.5 ml	Acrylamide 30%	400 µl
Bisacrylamide 1%	0.95 ml	Bisacrylamide 1%	250 µl
Tris-HCl 3M (pH 8.7)	0.95 ml	Tris-HCl 1M (pH 6.8)	315 µl
SDS 10%	75 µl	SDS 10%	25 µl
Deionized water	3.2 ml	Deionized water	1.5 ml
TEMED	5 µl	TEMED	2.5 µl
Ammonium persulfate 10%	100 µl	Ammonium persulfate 10%	25 µl
Final volume	7.78 ml	Final volume	2.52 ml

Table 6. Recipe for preparation of 10% polyacrylamide separating and 5% polyacrylamide stacking gels using a non-dissociating and discontinuous buffer system.

DNA ladder was also loaded in the gel. Electrophoresis was carried out in a BioRad cell in a non-dissociating and discontinuous buffer system (stacking gel buffer Tris-HCl 1M pH 6.8 and separating gel buffer Tris-HCl 3M pH 8.7). Both, in the stacking and separating gel solutions, 10% SDS was added in order to enhance electrophoretic resolution power (Day & Humphries, 1994). Electrophoresis was performed in running buffer pH 8.9 (0.3% Tris, 1.44% Glycine, 0.1% SDS) during 2hr at 60mA. The recipe used for discontinuous 10% polyacrylamide gel preparation is depicted in Table 6.

After electrophoresis, polyacrylamide gels were stained with silver nitrate following the Herring et al. (1982) method. It consisted of: i) fixation of the DNA fragments in 10% ethanol and 0.5% glacial acetic acid, ii) staining with 0.011M silver nitrate solution, iii) development with 0.75M NaOH and 7.6% formaldehyde, and iv) stopping the process with 5% glacial acetic acid when the desired image had developed. The duration of each step of the silver staining is shown in the Table 7.

Step	Solution	Duration
1	Fixing solution	30 min
2	Deionized water	2 min
3	Staining solution	30 min
4	Deionized water	10 sec
5	Developer solution	10-15 min (until bands are visible)
6	Stopping solution	Indefinitely

Table 7. Silver staining steps and duration.

After silver staining, polyacrylamide gels were dried and preserved. Each polyacrylamide gel was placed between two natural cellophane papers (one attached onto a glass) and immersed in a drying solution containing 69% methanol and 1% glycerol. Gels were dried at room temperature for 24-48hr (Giordano et al., 2008).

10. Results

10.1 Rotavirus G genotype detection by AGE/EBS and PAGE/SS

Under the described experimental conditions, the analysis by AGE/EBS of the 590 rotavirus positive samples showed that a total of 32 (5.4%) samples did not display a PCR G type amplification product after gel electrophoresis. Out of the 558 samples that revealed a PCR amplicon, 324 (58.1%) were single G genotype infections and 234 (41.9%) mixed G genotype infections (two or more amplicons were revealed in the same sample). On the other hand, PAGE/SS analysis of the PCR amplicons revealed that all the rotavirus positive samples (n=590) showed at least one amplicon. Out of the 590 samples, 318 (53.9%) were single G

Developing system	No. of rotavirus infection type		
	Single	Double	Triple
AGE/EBS	324	234	0
PAGE/SS	318	240	32

Table 8. Rotavirus infection type revealed by AGE/EBS and PAGE/SS.

genotype infections and 272 (46.1%) were mixed G type infections (240 double and 32 triple infections). It should be pointed out that, the total of the triple G genotype infections detected by PAGE/SS were developed as double or single G genotype infections by AGE/EBS. The results are depicted in Table 8.

The number of samples depicting each G genotype is shown in Table 9 and Figure 2. The results obtained showed that the standard AGE/EBS system revealed a lower number of genotypes than PAGE/SS.

Genotype	No. of detected genotypes by	
	AGE/EBS	PAGE/SS
G1	461	504
G2	46	88
G3	12	19
G4	253	255
G5	2	2
G8	2	3
G9	16	23
Total	792	894

Table 9. PCR products detection of rotavirus genotypes by AGE/EBS and PAGE/SS.

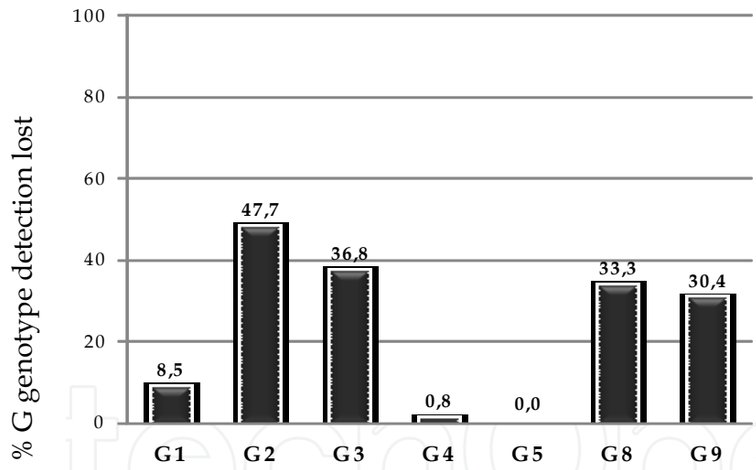

Fig. 2. Rate of rotavirus G genotype detection lost by AGE/EBS when compared with PAGE/SS.

11. Discussion

On the side-by-side comparison presented in this study, the amplicon detection methods revealed in general that a higher number of genotypes (11.4%) could be detected by PAGE/SS (n=894) than by AGE/EBS (n=792). In many cases, PCR products visualized as faint bands by PAGE/SS and later confirmed as specifics by nucleotide sequencing, were missed by the standard technique (AGE/EBS). Usually the common G1 and G4 genotypes were revealed as strong bands, while the other genotypes were often revealed as faint bands. Therefore, the tendency of AGE/EBS to detect a lower rate of G genotypes was even

more evident for the less frequent genotypes, that is G2, G3, G8 and G9. Moreover, 32 (5.4%) rotavirus positive samples did not revealed any PCR G type amplicon after AGE/EBS, meanwhile all of them were assigned to a G genotype after PAGE/SS.

In addition, the decreased in rotavirus genotype detection by AGE/EBS respect to PAGE/SS also impacted in the rate of mixed rotavirus infections. On the basis of these observations, it could be suggested that mixed G genotype infections rates reported worldwide, might be higher if the standard developing system, AGE/EBS, would be replaced by the PAGE/SS technique.

The frequent presence of multiple G genotypes in individual specimens may offer an unique environment for the reassortment of rotavirus genes. This notion highlights the need to improve methods allowing unveil rotavirus co-infections in future studies. These findings would be of interest in order to increase current knowledge about rotavirus evolution and determine the potential impact of mixed infections on rotavirus-vaccine coverage and vaccine efficiency.

Overall, the results obtained in this study highlight that the methodology employed for PCR products visualization could be an essential element for the description of the circulating rotavirus genotypes in a community and the rate of mixed G genotypes infections.

In view of the recent introduction of rotavirus vaccine in many countries, the correct identification of the G genotypes involved in the diarrheic illness and the match of the isolated G genotypes with those incorporated in the vaccine formulations are crucial for the accurate evaluation of rotavirus vaccine efficacy.

12. Acknowledgment

This work received financial support from the Council of Science and Technology of the National University of Cordoba, Argentina (Grant 2009-2010).

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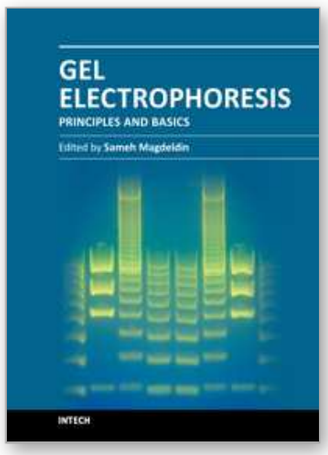

Gel Electrophoresis - Principles and Basics

Edited by Dr. Sameh Magdeldin

ISBN 978-953-51-0458-2

Hard cover, 346 pages

Publisher InTech

Published online 04, April, 2012

Published in print edition April, 2012

Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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