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EVALUATION OF PHENOTYPIC AND GENOTYPIC MARKERS FOR CLINICAL STRAINS OF *ACINETOBACTER BAUMANNII*

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Abstract *Acinetobacter baumannii* is an important opportunistic pathogen that is rapidly evolving toward multidrug resistance and is involved in various nosocomial infections that are often severe. It strongly prompts the epidemiological study of *A. baumannii* infections. However, there is no a generally accepted typing scheme. Different genotypic and phenotypic procedures were evaluated for the characterization of clinical isolates of *A. baumannii* isolated from a community hospital of Rosario, Argentina (Hospital de Emergencias Clemente Alvarez, HECA), during a period of four years. These included PCR with degenerate oligonucleotide primers (DO-PCR), repetitive extragenic palindromic-PCR (REP-PCR), pulsed-field gel electrophoresis (PFGE), and antibiotyping. Amongst individual methods, DO-PCR and PFGE were found the most suitable methods to discriminate *A. baumannii* clinical isolates [discriminatory indexes (D) of 0.98 and 0.96, respectively]. On the other hand, both antibiotyping and REP-PCR were much less discriminatory (D: 0.86 and 0.77, respectively). The combination of antibiotyping with any of the above genotypic procedures was found to largely increase D. In particular, the combination of DO-PCR and antibiotyping provided the best discriminatory method for epidemiological studies of *A. baumannii*. Combination of the different genotypic and phenotypic procedures allowed the inference of genetic relationships and dissemination of multidrug-resistant *A. baumannii* clones in HECA in the period 1994-1999. One particular strain, which showed sensibility to carbapenems, was found widely distributed in this hospital during 1994-1996. A different strain, showing additional resistance to carbapenems, rapidly disseminated in HECA in coincidence with the introduction of imipenem therapy in 1997.

Key words: molecular typing, nosocomial infections, *Acinetobacter baumannii*, genotypic markers

Resumen ***Evaluación de marcadores fenotípicos y genotípicos para cepas clínicas de* Acinetobacter baumannii.** *Acinetobacter baumannii* es un importante patógeno oportunista. Este microorganismo adquiere con facilidad resistencia a antimicrobianos, involucrándose en infecciones nosocomiales generalmente graves. Estas características promueven el análisis epidemiológico de las infecciones provocadas por el mismo. Sin embargo, no hay aún un esquema de tipificación generalmente aceptado para este patógeno. Hemos evaluado en este trabajo diferentes procedimientos fenotípicos y genotípicos para la caracterización de aislamientos clínicos de *A. baumannii* aislados en un Hospital Público de Rosario (Hospital de Emergencias Clemente Alvarez, HECA), durante un período de cuatro años. Estos incluyeron PCR con oligonucleótidos degenerados (OD-PCR), PCR empleando cebadores homólogos a secuencias palindrómicas extragénicas repetitivas (REP-PCR), electroforesis en geles de agarosa con campo pulsado (PFGE) y ensayo de susceptibilidad a antimicrobianos. OD-PCR y PFGE, entre los métodos individuales, fueron los métodos de mayor poder discriminatorio (índice discriminatorio, D, de 0.98 y 0.96; respectivamente). Por otra parte, el antibiotipo y REP-PCR presentaron menor discriminación (D: 0.86 y 0.77; respectivamente). La combinación del antibiotipo con cada uno de los procedimientos genotípicos mencionados originó un aumento importante en los índices discriminatorios de cada método. En particular, la combinación de OD-PCR y antibiotipo constituyó la mejor metodología para el estudio epidemiológico de *A. baumannii*. Así, la combinación de los procedimientos feno- y genotípicos mencionados permitió inferir las relaciones genéticas y la diseminación de clones de *A. baumannii* multirresistentes en el HECA en el período 1994-99. Una cepa particular, sensible a imipenem, estuvo ampliamente diseminada en el hospital durante 1994-1996. Por otra parte, un clon diferente, con resistencia adicional a carbapenemes, se diseminó rápidamente en el hospital en 1997, en coincidencia con la introducción de imipenem como terapia antibiótica.

Palabras clave: tipificación molecular, infecciones nosocomiales, *Acinetobacter baumannii*, marcadores genotípicos

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During the last years, *Acinetobacter baumannii* has increasingly become known as an agent of nosocomial infection[1]. They cause a wide range of clinical complications, such as pneumonia, septicemia, urinary tract infection, wound infection, and meningitis, especially in inmunocompromised patients[1]. This situation is aggravated by the rapid selection of multiple-drug resistant strains of this bacterium as a non-desired side effect of antimicrobial treatments[2]. The challenge posed by this emerging pathogen demands, among other responses, the use of discriminatory and reproducible typing methods to accurately characterize strains responsible of possible outbreaks, evaluate their persistence, and identify their routes of transmission[3]. A variety of typing methods have been tested for this purpose, including antibiotyping, plasmid profile, ribotyping, pulsed-field gel electrophoresis (PFGE), and PCR fingerprinting[4-10]. Yet, no generally accepted typing strategy has emerged for *Acinetobacter* strains from these studies, although PCR fingerprinting is generally employed for typing procedures due to its simplicity and rapidity[11-13]. Still, a comparative evaluation of different techniques is always recommended in the sense that it identifies the particular methodology or methodologies most suited for epidemiological purposes[7-9].

We have evaluated different methods aimed to characterize *A. baumannii* isolates from a community hospital of Rosario, Argentina, including PCR fingerprinting, repetitive extragenic palindromic-PCR, pulsed-field gel electrophoresis, and antibiotyping. A combination of these procedures was also employed for the inference of clonal relationships amongst multidrug-resistant *A. baumannii* strains causing a variety of infections during 1994-1999 in the above nosocomial institution.

Materials and Methods

Bacterial isolates

Bacterial isolates were obtained from colonized or infected patients during November 1994 through March 1999 in Hospital of Emergencies Clemente Alvarez (HECA), Rosario, Argentina. HECA is a public, 150-bed tertiary-care teaching hospital with an average attendance of around 35000 patients per year. The intensive-care unit of this hospital is constituted by a 10-bed facility, admitting an average of around 700 patients per year.

Identification of bacterial isolates as *A. baumannii* was performed by the API 20NE Identification System (*bioMérieux*, Lyon, France) and by their ability to grow at 37°C, 41°C, and 44°C[4]. Relevant data of the 42 isolates employed in this study are provided in Tables 1 and 2.

Antibiotype patterns

The susceptibility of *A. baumannii* isolates to different antimicrobial agents was assessed by the disk diffusion method on Mueller-Hinton agar[14] (Table 2). In total, 11 antimicrobials were tested, which were classified in 5 different groups as follows: group 1 comprised meropenem and imipenem; group 2, other β-lactams including ampicillin-sulbactam, ceftazidime, cefota-xime, piperacillin, and piperacillin-tazobactam; group 3, aminoglucosides including gentamicin and amikacin; group 4, ciprofloxacin; and group 5, trimethoprim-sulfamethoxazole. Bacterial isolates were assigned to different antibiotypes (I-VIII, Table 2) on the basis of the following criteria: two isolates were considered to belong to a different antibiotype when the diameters of the inhibition zones corresponding to four or more of the above antimicrobials showed differences higher than 3 mm (data not shown).

Isolation of Acinetobacter baumannii *DNA*

Bacterial DNA was obtained from cells treated with lysozyme/proteinase K, followed by phenol extraction to remove contaminating proteins[15]. DNA concentrations were determined spectrophotometrically[15].

PCR fingerprinting

A PCR assay employing degenerate oligonucleotide primers (DO-PCR) was employed, following essentially previously described methods[16]. *A. baumannii* isolates were considered different when their amplification profiles differed in more than two bands. Differences in band intensity at a given position between two given isolates were not considered significant for their differentiation.

REP-PCR reactions were performed essentially as previously described[9]. A difference of 1 or 2 bands was not considered for discrimination purposes, providing that all other bands migrated at equivalent positions[9].

Pulsed-field gel electrophoresis

Bacterial DNA was prepared and digested with *Apa*I restriction endonuclease essentially as described[17]. The resulting fragments were separated in a 1% agarose gel employing a *CHEF-DR II Bio-Rad* apparatus (*Bio-Rad Laboratories*, Richmond, CA) at 4.5 V/cm by using the following two intervals of ramped pulse times: 1 to 10 s for 12 h and 10 a 100 s for 16 h. PFGE patterns were interpreted following Tenover et al[18]. Two given isolates were considered different if their PGFE patterns differed in at least four bands[18].

Data acquisition and analysis.

DNA fingerprints generated by the different methods were examined visually, and a numeric profile for each strain was constructed on the basis of the presence or absence of an amplification band at a given position[19]. A similarity matrix was calculated from these data following the procedure of Nei and Li[20]. In turn, this matrix was used to generate a dendrogram according to the neighbor-joining method[21].

Discriminatory index

A numerical index of discrimination (D)[22] was used to evaluate the discriminatory power of each individual method or their different combinations.

Results

As noted above, 8 different antibiotypes were defined amongst the 42 *A. baumannii* isolates analyzed in this

TABLE 1.– *Characterization* of Acinetobacter baumannii *isolates included in this study*

N	Isolate	Date	Ward*	Source†	ATB‡	DO-PCR type	REP-PCR type	PFGE§ type
1	8265	11/94	4	CSF	I	A	A’	ND
2	8795	12/94	1A	urinary tract	II	A	A’	ND
3	8808	12/94	4	urinary tract	III	B	B’	B’’
4	8822	12/94	1A	CSF	I	A	A’	ND
5	2339	03/95	Burns	blood	IV	C	C’	C’’
6	2390	04/95	1A	CSF	I	A	A’	ND
7	1206	06/95	1A	wound	I	A	A’	ND
8	1259	06/95	1A	pleural fluid	I	A	A’	ND
9	2371	07/95	1A	CSF	II	A	A’	ND
10	3289	08/95	1A	catheter	I	A	A’	ND
11	3851	09/95	1A	blood	I	A	A’	ND
12	5005	10/95	1A	wound	I	A	A’	ND
13	5922	11/95	1A	catheter	I	D	B’	D’’
14	7233	01/96	1A	blood	II	A	A’	ND
15	11604	06/96	ITU	BAL	I	A	A’	ND
16	11638	06/96	1A	pleural fluid	I	A	A’	ND
17	11959	07/96	ITU	blood	I	A	A’	ND
18	12690	08/96	3	bone	I	A	A’	ND
19	15034	10/96	ITU	CSF	II	A	A’	ND
20	15971	11/96	ITU	CSF	II	A	A’	ND
21	22920	8/97	1A	urinary tract	V	B	B’	E’“
22	23395	9/97	1B	CSF	I	A	A’	A’’
23	24214	10/97	2	AF	V	B	B’	E’“
24	24280	10/97	OPD	urinary tract	V	B	B’	E’’
25	24419	10/97	OPD	catheter	VI	E	D’	F’’
26	24457	10/97	ITU	CSF	V	B	B’	E’’
27	24474	10/97	ITU	blood	I	F	B’	G’’
28	24578	10/97	ITU	wound	V	B	B’	E’“
29	24664	10/97	1B	biopsy	V	B	B’	E’’
30	25365	10/97	1A	pleural fluid	VII	G	E’	H’’
31	26597	1/98	4	wound	V	B	B’	ND
32	28616	3/98	2	BAL	II	A	F’	D’’
33	28316	3/98	1B	urinary tract	V	B	B’	ND
34	28435	3/98	2	blood	V	B	B’	E’’
35	28871	4/98	OPD	wound	I	H	B’	B’’
36	29345	4/98	4	bone	I	F	B’	G’’
37	29408	4/98	ITU	BAL	II	A	F’	D’’
38	29533	4/98	OPD	catheter	VI	I	G’	I’’
39	29697	5/98	ITU	wound	I	J	B’	A’’
40	29721	5/98	1A	blood	VI	K	H’	J’’
41	31082	6/98	2	wound	V	B	B’	E’’
42	825	3/99	ITU	wound	VIII	L	B’	K’’

**ITU: Intensive Therapy Unit; OPD: Out-patients Department*
†CSF: cerebrospinal fluid; BAL: bronchoalveolar lavage; AF: ascitic fluid
‡ATB: antibiotype (see Materials and Methods).
§ND: No determined.

TABLE 2.– *Antimicrobial susceptibility patterns of* Acinetobacter baumannii *strains*

		Antimicrobial assayed‡										
		Carbapenems		β-lactams					Aminoglucosides		QN	Others
Atb*	Description†	MER	IMP	AMS	CAZ	CTX	PIP	PIP-Taz	GEN	AKN	CIP	TMS
I	MR	S	S	V	R	R	R	R	R	R	R	R
II	MR	S	S	S	S	R	R	S	S	R	R	R
III	MRImp^{I}	I	I	S	R	R	R	R	R	R	R	R
IV	S	S	S	S	S	S	S	S	S	S	S	S
V	MRImp^{R}	R	R	V	S	R	R	R	R	R	R	R
VI	S	S	S	S	S	I	S	S	S	S	S	S
VII	S	S	S	S	S	S	R	S	R	S	S	R
VIII	MRImp^{R}	R	R	S	R	R	R	R	R	R	R	S

* *Criteria for antibiotype (Atb) characterization were defined in Materials and Methods.*

† *Isolates were considered multidrug-resistant (MR) when they expressed resistance to at least three of the antimicrobial groups defined (see Materials and Methods), and susceptible (S) when they showed susceptibility to at least three groups (in both cases with the exclusion of the carbapenems). A given isolate was considered resistant to a particular antimicrobial group when it expressed resistance to at least half of the antimicrobials that composed the group. MRImp^{I} or MRImp^{R} strains correspond to multidrug-resistant strains with intermediate or resistant category for imipenem, respectively*[14].

‡ *QN: quinolones. MER: meropenem; IMP: imipenem; AMS: ampicillin-sulbactam; CAZ: ceftazidime; CTX: cefotaxime; PIP: piperacillin; PIP-Taz: piperacillin-tazobactam; GEN: gentamicin; AKN: amikacin; CIP: ciprofloxacin; TMS: trimethoprim-sulfamethoxazol; S: susceptible; R: resistant; I: intermediate; V: variable*[14].

Fig. 1.– Characterization of *A. baumannii* clinical isolates by DO-PCR. All procedures are described in Materials and Methods. The following isolates of *A. baumannii* were used (see Table 1 for details): Lane 1, 23395 (A); lane 2, 24214 (B); lane 3, 5922 (D); lane 4, 29345 (F); lane 5, 29697 (J); lane 6, 28871 (H); lane 7, 825 (L); lane 8, 2339 (C); lane 9, 25365 (G); lane 10, 29533 (I); lane 11, 29721 (K); lane 12, 24419 (E). The pattern assigned to each isolate is indicated between brackets. In some cases, the amplification patterns correspond to an isolate representative of the clon (Table 1). MR: multiresistant isolates; S: susceptible isolates. The position of the size markers (*Eco*RI/*Hind*III-digested lambda DNA) are indicated in the left margin.

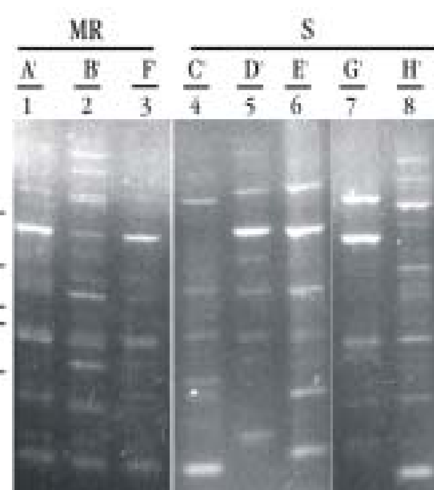

Fig. 2.– Characterization of *A. baumannii* clinical isolates by REP-PCR. All procedures are described in Materials and Methods. The following isolates were used (see Table 1 for details): Lane 1, 23395 (pattern A'); lane 2, 28871 (B'); lane 3, 28616 (F'); lane 4, isolate 2339 (C'); lane 5, isolate 24419 (D'); lane 6, isolate 25365 (E'); lane 7, 29533 (G'); lane 8, 29721 (H'). For details see the legend to Fig. 1.

work (Tables 1 and 2). Five of them were found amongst the 37 multidrug-resistant (MR) isolates (*i.e.*, I, II, III, V, and VIII), and 3 amongst the 5 susceptible (S) isolates (*i.e.,* IV, VI, and VII).

The use of DO-PCR analysis disclosed the presence of twelve distinct patterns among the same isolates (A-L, Fig. 1 and Table 1). Interestingly, the 5 S strains showed 5 different band profiles (C, E, G, I, and K, respectively). A lower degree of diversity was observed amongst the 37 MR isolates, which showed the presence of seven different patterns (A, B, D, F, H, J, and L). It is worth noting at this stage that 17 out of the 19 MR isolates recovered in HECA during the period 1994-1996 (*i.e.*, 89%) belonged

to DO-PCR type A, whilst only 1 strain was found to possess profile B during the same period (Table 1). On the other hand, 10 out of the 18 (*i.e.*, 56%) MR strains isolated in the period 1997-1999 belonged to genotype B, while the number of isolates retaining profile A had been reduced to only 3 (*i.e.*, 17%) of the analyzed sample during the same period (Table 1). These results suggested that different clones disseminated in HECA during each particular period.

The analysis of the same bacterial sample by REP-PCR indicated the existence of eight different patterns (A’ to H’, Fig. 2 and Table 1). Again, all 5 S isolates were clearly differentiated by this procedure (patterns C’, D’, E’, G’, and H’, respectively, Table 1). As above, the number of different genotypes was much reduced for MR isolates (Table 1). However, REP-PCR showed the presence of only three distinct profiles amongst the MR strains (A’, B’, and F’, Table 1), contrasting the results of DO-PCR (see above).

The same strains were further analyzed by PFGE. By this procedure, eleven different patterns were disclosed (A’’ to K’’, Fig. 3 and Table 1). Again, 5 of them corresponded to S isolates (C’’’, F’’’, H’’’, I’’’, and J’’’, respectively, Table 1), whereas six differentiated patterns were observed amongst the MR isolates (A’’’, B’’’, D’’’, E’’’, G’’’, and K’’’, Table 1).

The number of types and discriminatory indexes calculated for each particular procedure and for their combination are summarized in Table 3. As seen in the table, OD-PCR and PFGE showed the highest discriminatory capabilities (D of 0.98 and 0.96, respectively). On the other hand, antibiotyping and REP-PCR were much less discriminatory (D of 0.86 and 0.77; respectively). Interestingly, the combination of antibiotyping with any of these genotypic procedures largely increased discriminatory indexes (Table 3).

A dendrogram was constructed on the basis of the combined data of DO-PCR, REP-PCR and PFGE for 14 representative *A. baumannii* strains recovered in the pe-

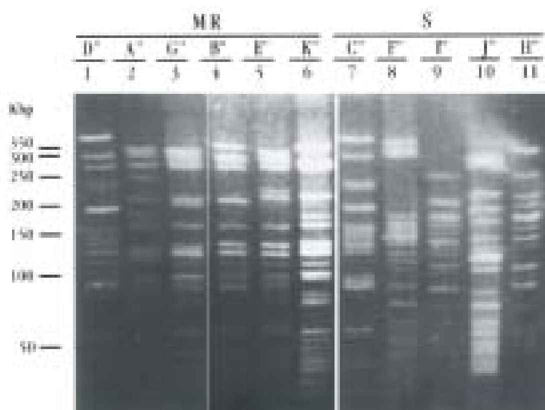

Fig. 3.– Characterization of *A. baumannii* clinical isolates by PFGE. The following isolates were used: lane 1, 5922 (pattern D’’); lane 2, 29697 (A’’) ; lane 3, 24474 (G’’); lane 4, 28871 (B’’); lane 5, 24214 (E’’); lane 6, 825 (K’’); lane 7, 2339 (C’’); lane 8, 24419 (F’’); lane 9, 29533 (I’’); lane 10, 29721 (J’’); lane 11, 25365 (H’’). For other details see the legend to Fig. 1.

TABLE 3.– *Discriminatory indexes of the phenotypic and genotypic techniques used in this study**

Typing method	Number of types	Discrimination index (D)
Antibiotyping (ATB)	8	0.86
DO-PCR	12	0.98
REP-PCR	8	0.77
PFGE	11	0.96
DO-PCR+ ATB	14	1.00
DO-PCR + PFGE	14	1.00
PFGE + ATB	13	0.99
REP-PCR + ATB	11	0.93

**D estimations were done employing* A. baumannii *isolates 8808, 2339, 5922, 23395, 24214, 24419, 24474, 25365, 28616, 28871, 29533, 29697, 29721 and 825 (see Table 1).*

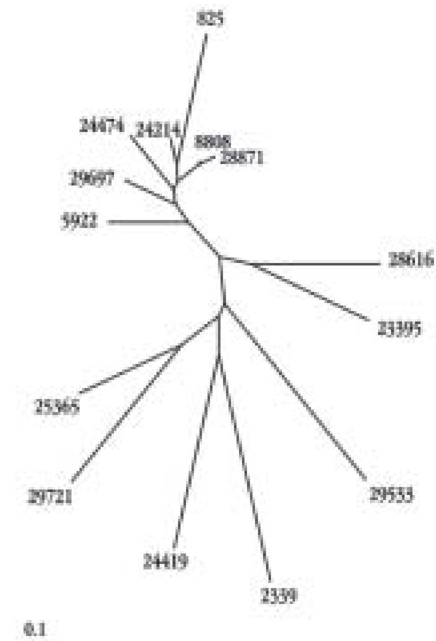

Fig. 4.– Dendrogram indicating genetic relationships between relevant *A. baumannii* strains analyzed in this study. The dendrogram was inferred from a distance matrix constructed from the combined data of DO-PCR, REP-PCR and PFGE of fourteen bacterial isolates showing representative patterns. For details see Materials and Methods.

riod November 1994 through March 1999 (Fig. 4). As seen in the figure, the S isolates appeared clearly differentiated from the MR strains, composing a separate and highly divergent group. In turn, the more compact clustering of the MR strains suggests a closer genetic relationships. In particular, a cluster of highly related isolates was noteworthy in that it contained strains that showed different levels of resistance to imipenem (Fig. 4, see also Table 2).

Discussion

Since 1994, significant increases in the recovery of multiresistant *A. baumannii* clinical strains was noted in HECA, particularly amongst immunocompromised and intensive-care unit patients. This resulted in considerable overuse of imipenem, to which the organisms were uniformly susceptible. In 1997, carbapenem-resistant strains emerged and rapidly disseminated, prompting us to conduct this epidemiological investigation. Initial screenings were done by determining antibiotic resistance patterns (Tables 1 and 2), in an attempt to identify the dissemination of particular strains. However, attempts to perform epidemiological studies soon required the use of more discriminatory markers than those provided by antibiotyping. We therefore evaluated different genotypic procedures, including DO-PCR, REP-PCR and PFGE, in order to analize their usefulness to characterize *A. baumannii* isolates obtained in the period from November 1994 through March 1999.

In our experience, DO-PCR performed as the most discriminatory amongst all tested methods (D=0.98), even slightly higher than the most-elaborated PFGE technique (D=0.96). On the other hand, the performance of REP-PCR was much poorer (D=0.77), even lower than antibiotyping (D=0.86). Interestingly, the combination of antibiotyping with any of the above genotypic methodologies increased discriminatory capability (Table 3). In this sense, the best discrimination was provided by the combination of DO-PCR with antibiotyping (D=1, Table 3). This indicates that antibiotype may still provide a valuable tool for epidemiological purposes.

The combination of all genotypic markers was employed to determine the population structure of *A. baumannii* during this period (Fig. 4). As expected, MR strains showed much less genetic variability than S strains, most probably reflecting the selective pressure caused by antimicrobial therapy. In addition, this analysis suggested that a particular epidemic MR *A. baumannii* strain disseminated in HECA during the period 1994-1996 (clone A, Fig. 1 and Table 1). This clone was replaced since 1997 by an emerging new clone with additional resistance to imipenem (clone B), a situation that was coincident with the introduction of this antibiotic therapy. An infection control strategy that included strict compliance with cross transmission prevention protocols and restrictions in the use of carbapenem was implemented after this study, resulting in a marked reduction in the incidence of infection and colonization by MR *A. baumannii* strains.

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Some time in the 1970s, as happened in physics half a century before, the old world of certainty, stability and determinism in biology fell. In its place we must build a world of fluctuation, change and unpredictability. The genome that we decipher in this generation is but a snap-shot of an ever-changing document. There is no definitive edition.

En un momento determinado de los años setenta, tal como ocurrió con la física medio siglo antes, cayó el viejo mundo de la certidumbre, la estabilidad y el determinismo biológico. En su lugar debemos construir un mundo de fluctuación, cambio e incertidumbre. El genoma que desciframos en esta generación no es más que la instantánea de un documento que cambia constantemente. No hay una edición definitiva.

Matt Ridley

Genome: the autobiography of a species in 23 chapters. New York: Harper Collins, 1999, p 146
(Genoma: la autobiografía de una especie en 23 capítulos. Traducción de Inés Cifuentes. Madrid: Grupo Santillana de Ediciones S.A., 2000, p 170)