Virus mutations and their impact on vaccination against infectious bursal disease (Gumboro disease)

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Summary

Infectious bursal disease (also known as Gumboro disease) is an immunosuppressive viral disease specific to chickens. In spite of all the information amassed on the antigenic and immunological characteristics of the virus, the disease has not yet been brought fully under control. It is still prevalent in properly vaccinated flocks carrying specific antibodies at levels normally high enough to prevent the disease.

Trivial causes apart, failure of vaccination against infectious bursal disease is associated mainly with early vaccination in flocks of unknown immune status and with the evolution of viruses circulating in the field, leading to antigenic drift and a sharp rise in pathogenicity. Various highly sensitive molecular techniques have clarified the viral determinants of antigenicity and pathogenicity of the infectious bursal

disease virus. However, these markers are not universally recognised and tend to be considered as evolutionary markers.

Antigenic variants of the infectious bursal disease virus possess modified neutralising epitopes that allow them to evade the action of maternally-derived or vaccine-induced antibodies. Autogenous or multivalent vaccines are required to control antigenic variants in areas where classical and variant virus strains coexist. Pathotypic variants (very virulent viruses) remain antigenically related to classical viruses. The difficulty in controlling pathotypic variants is linked to the difficulty of eliciting an early immune response, in view of the risk of the vaccine virus being neutralised by maternal antibodies. Mathematical calculation of the optimal vaccination time and the use of vaccines resistant to maternally-derived antibodies have improved the control of very virulent viruses.

Keywords

Antigenic variant – Gumboro disease – Infectious bursal disease – Mutation – Pathotypic variant – Vaccination failure – Virus.

Introduction

Infectious bursal disease (IBD), also known as Gumboro disease, is a highly contagious viral disease that preferentially targets the bursa of Fabricius of young chickens. The financial impact of the disease is difficult to quantify because of the insidious nature of its immunosuppressive form, making poultry more susceptible to the most innocuous pathogens. This has the effect of encouraging greater use of antibiotics, which contributes to the development of antimicrobial resistance harmful to human and animal health.

The virus responsible for IBD consists of two segments of double-stranded ribonucleic acid (RNA), which has no envelope, rendering it highly resistant to the outside environment. A serum neutralisation test can be used to distinguish between the two serotypes of the IBD virus (IBDV): 1 and 2 (1), which provide no cross-protection

in vivo (2). All the viruses causing clinical and sub-clinical disease belong to serotype 1. Serotype 2 strains are non-pathogenic (3).

The viral RNA comprises two segments: A and B. Segment B is monocistronic and encodes protein VP1, which is none other than the viral RNA polymerase. Segment A is polycistronic and encodes two major structural proteins (VP2 and VP3); one protease (VP4) responsible for cleavage of the viral polyprotein; and one non-structural protein (VP5), which is expressed transiently at the end of the virus life cycle and is believed to be responsible for breaking down the membrane of the infected cell (4), releasing the viral particles.

Protein VP2 contributes to the antigenicity (5), tropism and pathogenicity of the virus (6, 7, 8). It forms trimeric sub-units to build the outer structure of the viral capsid and exhibits surface projections corresponding to the central (or hypervariable) region of the VP2 protein. This is the part of the protein most exposed to immune pressure and hence the one most prone to mutations.

The VP2 hypervariable region comprises four domains with loop structures. Domain PBC, situated between amino acid positions 219 and 224, is believed to be responsible for stabilising the conformation of epitopes, while domain PHI (amino acids 315-324) is thought to be involved in recognition by neutralising antibodies (9). Amino acid substitutions in domain PHI, probably by causing conformational changes in viral epitopes, are believed to allow the virus to evade the immune response of even the most immunised poultry (9, 10). The two additional domains − PDE (amino acids 250–254) and PFG (amino acids 283–287) – include residues 253 and 284, respectively, which are implicated in the infectivity of cell cultures and virus pathogenicity (6).

Viral diversification mechanisms of RNA viruses

A common genetic diversification mechanism for RNA viruses is the creation of new virus strains. Recent studies have estimated that the mutation rate per nucleotide across the hypervariable region of

IBDV protein VP2 is close to 0.24% (11). It is believed to be ten times lower for rotavirus, another segmented RNA virus.

Certain mutations that occur during the IBDV life cycle are incompatible with survival of the virus. In contrast, other mutations enhance the ability of the virus to multiply in the chicken's body. This means that mutants formed as a result of transcription errors during viral replication undergo positive selection dictated by a variety of environmental factors, the most important of which is vaccine pressure (12, 13).

Just as with influenza viruses, the segmented genome of IBDV theoretically allows genetic reassortment between related virus strains during co-infection (14, 15). In particular, this may occur when chickens are vaccinated with a live vaccine while incubating a wild-type virus. A compatibility control mechanism between segments could promote co-evolution of the two segments by limiting the formation of reassortant viruses. Such control could involve, for example, interactions between protein VP3 encoded by segment A and protein VP1 encoded by segment B (16).

Le Nouën *et al.* (15) confirmed the co-evolution of segments A and B after conducting molecular characterisation and phylogenetic analysis of 50 isolates. However, 13 of the 50 strains examined were identified as exhibiting a different phylogenetic position depending on which segment was analysed. Use of the Basic Local Alignment Search Tool (BLAST) to search segments A and B of strain KZC-104 (isolated in Zambia in 2004) revealed more than 98% nucleotide sequence identity to segment A of the very virulent strain D6948 and nearly 99.8% identity to segment B of attenuated strain D78 (17). This confirms the possibility of segment reassortment under natural conditions. More recently, a study of the electrophoretic profile of digestion products demonstrated the different origin of segments A and B of strain Gx (18). This supports the results of sequence analyses of the same strain by Gao *et al.* (19) five years earlier. The incongruous results of certain phylogenetic analyses of IBD field isolates leave no doubt as to the

existence of genetic recombination between homologous virus strains. He *et al.* (20) identified breakpoints at positions 636 and 1743 in segment A of strain KSH. The sequences outside this fragment appear similar to those characterising attenuated vaccine strains, while the sequences inside the fragment are similar to those of very virulent viruses. Certain strains isolated in Latin America between 2001 and 2011 exhibited, within the VP2 hypervariable region, a combination of amino acids taken from the variant and classical viruses (21) (Fig. 1). Natural homologous recombination can distort the results of phylogenetic analyses and must therefore be considered in any epidemiological study.

The evolution of IBD viruses, by point mutation, genetic reassortment or homologous recombination, has led to the emergence of antigenic and pathotypic virus variants. Antigenic variants were first described in the United States of America (USA) in 1984 (22). Since then, they have been reported in Canada (23), Australia (24) and Central and South America (25). Wild-type viruses, exhibiting atypical antigenicity, have been identified in Europe (5, 26). Very virulent viruses were first reported in Europe in 1987 (27). Nowadays they are distributed worldwide, with only Australia and New Zealand remaining free (Fig. 2).

Viral determinants of antigenicity

Serotype 1 IBD viruses are divided into two antigenic groups – classical and variant – each carrying typical amino acids, which mutate frequently to form virus sub-types within the two antigenic groups.

The antigenic phenotype is determined primarily by the VP2 hypervariable region (amino acids 206–350: restriction fragment AccI-SpeI) in segment A. A study based on the use of a battery of monoclonal antibodies has identified the missing or altered epitopes on the surface of variant viruses isolated in the USA. For instance, epitope B69, which is found on the surface of all classical viruses (except vaccine strain PBG98), proved to be absent from the Delaware variants (A, D, G and E). The loss of epitope B69 is

believed to be linked to Q249K substitution (30). However, the ability of strain Bel-IBDV to bind monoclonal antibody B69 with residue H at this position suggests that several amino acids at position 249 can accommodate monoclonal antibody B69 (5).

A second epitope, designated R63, which is usually found in both the Delaware variants and serotype 1 classical viruses, was absent from 50% of the 319 viruses isolated in the Delmarva region of the USA (31). These isolates formed a new population of variants known as GLS variants. The same study distinguished another population of variants, designated DS326. The DS326 variants have lost not only epitopes B69 and R63 but also epitope 179, which is found on the surface of both the Delaware and GLS variants. The GLS and DS326 variants share epitope 57, while the E/Delaware (E/Del) variant is characterised by possessing epitopes 67 and BK9 (Table I).

Sequence alignment between variant and classical viruses has identified different residues at positions 222, 249, 286 and 318 (32). More recently, birds vaccinated with the E/Del variant could not be protected against a challenge with variants E/Del-222 and E/Del-254, two viruses that are absolutely identical to E/Del except at positions 222 (T222A) and 254 (S254N), respectively (33). This confirms that a single point mutation can lead to antigenic drift allowing the mutant virus to escape the immunity conferred by the parental virus.

Relative to the amino acid sequence in the VP2 domain of classical viruses, that of variant A includes six residue substitutions (34), while those of variants E/Del, GLS and DS326 include one or two substitutions in each of the two major VP2 hydrophilic regions (PBC and PHI) (9, 35). All these variants include the substitution Q249K (36). The existence of specific nucleotide sequences of pathotypes allows the use of probes to identify and differentiate between classical, variant and very virulent viruses by means of quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (37).

One study of the antigenicity of IBD viruses also analysed restriction-enzyme maps (38). However, the results of such an analysis serve

only as a guide, because they are prone to distortion from synonymous mutations with no effect on viral phenotype.

Viral determinants of virulence

The specific viral determinants of virulence uncovered by various studies are not universally recognised. Sequencing of the A and B segments of pathogenic strains (serotype 1) and non-pathogenic strains (serotype 2) has revealed different nucleotides along the two segments, suggesting multigene virulence. However, between pathogenic and non-pathogenic strains, there is a smaller degree of nucleotide identity within segment A than within segment B (39). This low genetic identity between serotype 1 and 2 viruses in segment A is attributed mainly to the sequence encoding the hypervariable region of structural protein VP2.

Even though amino acid changes within VP2 form the molecular basis of the pathotypic variations, VP2 is not the only marker of IBDV pathogenicity. Indeed, substitution of the VP2 gene in attenuated strain CEF94 with the same gene in very virulent strain D6948 did not lead to an increase in the pathogenicity of the attenuated strain (40).

The most commonly used methods for identifying the viral determinants responsible for virulence are: the creation of chimeric viruses; the study of virus reactivity to monoclonal antibodies; and the alignment of amino acid sequences within the viral proteins of the different IBDV strains.

Unlike classical and attenuated virulent strains, very virulent viruses, particularly those of European origin, react weakly with monoclonal antibodies 3 and 4 (Table II), with which binding involves amino acids 222P and 223G (26) (Table III). Moreover, only the virulent strains appear to react with monoclonal antibody 21 (42). Chicken recombinant antibody 88 (CRAb 88) reacts solely with highly pathogenic viruses from a variety of origins. Residues 294I and 256I, specific to very virulent viruses, appear to play a role in binding of CRAb 88 (43).

A search for the restriction sites of different enzymes in PCR products has been used to differentiate the various virus pathotypes (38, 44). In an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA), the BspMI restriction site, corresponding to substitution P222A, was responsible for loss of neutralisation in the monoclonal antibodies of very virulent viruses and has been proposed as a marker of very virulent strains (26). Very virulent strains also share the SspI restriction site, which is often used to characterise highly pathogenic viruses (44). One exception is very virulent West African strain 88180, which lacks both the BspMI and SspI sites (45).

A more recent study identified, in the sequence encoding the VP5 non-structural protein, a unique TfiI restriction site located exclusively in viruses that are not very virulent (i.e. classical, attenuated, variant and serotype 2 viruses) (46).

The advantage of using monoclonal antibodies over a restriction map using endonucleases is that antibodies are more reliable. The epitope identifying the monoclonal antibody is dictated directly by encoded amino acids and, unlike restriction sites, cannot be affected by possible silent mutations.

Sequence alignment of amino acids from the virulent, avirulent and attenuated strains has also identified mutations associated with the pathogenicity of IBD viruses. All but one of the residue substitutions found in highly pathogenic viruses are located in the VP2 hypervariable domain (47).

The co-existence of the two amino acids 279D and 284A determines the virulent phenotype (48) (Fig. 3). Substitutions of these two residues would lead to loss of hydrophilicity of the VP2 hypervariable region and could reconfigure the virion surface structure. A point of note is that the 279D residue is well conserved in two non-pathogenic strains of serotype 2: OH and 23/82. Similarly, virulent strains GLS and Cu-1 share the T284 residue with naturally non-pathogenic strains of serotype 2 (36).

The sequence thought to be involved in virulence is the heptapeptide adjacent to the second hydrophilic region of the VP2 domain (amino acids 326–332) (49), which, in the very virulent Japanese and European viruses and the USA antigenic variants, invariably includes the serine-rich conserved sequence S-W-S-A-S-G-S (39, 50). Nevertheless, very virulent strain OKYM conserved the serine-rich heptapeptide even after being attenuated (strain OKYMT) (48).

The most virulent strains would appear to be those with the greatest serine residues in this region of the VP2 domain (30). The hydrogen bonds present in the serine-rich motif would appear to allow intra- and inter-molecular interactions, which are crucial for virulence. In non-pathogenic or low-pathogenic viruses, such interactions are impossible because substituting one or two serines (S) with other residues would take up more space in the structure (9).

Not only do the Japanese and European highly pathogenic viruses share the serine-rich sequence, they all share, simultaneously, unique amino acids at positions 222, 256 and 294, which are not found in less virulent viruses (45).

Impact of virus mutations on vaccination efficacy

Until the mid-1980s, IBD was kept fully under control by vaccination and caused no more than 2% specific mortality in broiler flocks. Since then, vaccination failures have become ever more common and mortality rates have been rising all the time. This new trend is linked closely with the emergence of antigenic variants and pathotypic variants (very virulent viruses). Even though none have yet been reported, the emergence of highly pathogenic antigenic variants should not be ruled out, which would make poultry vaccination even more complex.

Antigenic variants cannot be controlled by conventional serotype 1 vaccines because of antigenic distance between field strains and vaccine strains. Between variant and classical viruses there are transitional viruses (51) that may not undermine vaccination efficacy.

The most important requirement for the production of vaccines against viruses displaying antigenic diversity is a method for measuring antigenic distances between strains and for ascertaining how these distances relate to cross-protection. Antigenic cartography, a new computational method for quantifying antigenic distances between strains, has been applied to human and equine influenza viruses to examine the evolution of these viruses in relation to vaccine strains (52). This method is highly applicable to other antigenically variable pathogens, including IBDV.

While epidemiological studies in a number of countries, notably the USA, have demonstrated wide antigenic diversity among wild-type viruses in circulation, the vaccines currently on the market are far from able to match this diversity. Even though variant strains carry modified neutralising epitopes, cross-protection has been obtained experimentally between variant and classical viruses. While this protection was only partial, it was improved by increasing vaccine antigenic mass and/or dose (53). The viral dose of the challenge is also critical. Increasing the dose leads to a significant decrease in cross-protection (54). Multivalent recombinant vaccines with a broader antigenic spectrum have afforded complete protection against variant and classical virus challenges (55).

Autogenous vaccines are also offered as a solution to vaccination failures associated with antigenic variants. The first step in the selection of candidate autogenous vaccines is to analyse nucleotide sequences in the viruses responsible for such failures and to confirm changes in neutralising epitopes. High-resolution melt analysis of amplicons obtained by quantitative RT-PCR can be very useful in developing autogenous vaccines (56), as it allows faster and cheaper detection of point mutations in the viral genome of wild-type viruses implicated in vaccine failure in a given region.

It is imperative to recognise that only a vaccination-challenge can identify true antigenic variants. However, new information on the crystallographic structure of structural protein VP2 suggests that antigenic drift can be predicted by studying the three-dimensional

molecular structure of the isolate (57). It is now possible to determine whether an amino acid mutation will be buried inside the molecule or exposed on the surface of the VP2 protein. Surface mutations are more likely to change the antigenicity and contribute to antigenic drift.

The difficulty in controlling pathotypic variants (very virulent viruses) is, in turn, linked to the difficulty of eliciting an early immune response in chickens, rather than to the existence of antigenic drift among wild-type viruses. Contrary to antigenic variants, cross-protection exists between very virulent viruses and classical serotype 1 virulent viruses (58), including attenuated vaccines. However, antigenic differences were demonstrated among vaccine strains. These differences are attributed to a process of attenuation of live vaccines, which creates a heterogeneous population of viruses with antigenic properties, some of which are sufficiently modified to inhibit protection against circulating very virulent viruses. Under experimental conditions, the Winterfield strain failed to protect against challenge from a very virulent virus (59). This point to a need for molecular characterisation of vaccine viruses to minimise the risk of antigenic drift.

Before the advent of very virulent viruses, passive immunity transferred to chicks was sufficient to provide protection for at least three to four weeks. Nowadays, increasing pathogenicity among wild-type viruses has lowered the age of susceptibility of chicks, making early infection (before 21 days of age) entirely possible. It is important, therefore, that the decaying passive immunity in chicks be offset with active immunity as soon as possible. This was the thinking behind the approach based on an optimal choice of vaccination time for proper vaccine take in order to ensure an effective bridge between maternally derived antibodies and vaccine-induced antibodies without the risk of vaccine neutralisation (60). This risk can also be averted by administering immune-complex vaccines (resulting from attenuation of the virus under immune pressure) or turkey herpesvirus recombinant vaccines expressing the VP2 protein (HVT-VP2) (resulting from the use of expression vectors of viral genetic material). These vaccines have the advantage of being safe (61, 62), insensitive to

maternal antibodies (63, 64) and can be administered *in ovo* or at one day of age.

Vaccination with recombinant vaccine HVT-VP2 prevents the development of an immunity gap (62) and ensures long-lasting presence of the viral protein (65). As turkey herpesvirus is non-pathogenic for chickens, its administration is likely to induce persistent infection in the host. Under experimental conditions, even though the recombinant vector vaccine expressed the viral protein of classical strain F 52/70 (66), it afforded protection against the USA variant E/Del (64, 67).

The mechanism of action of immune-complex vaccines has yet to be fully established. However, their advantages over conventional vaccines are thought to be linked to their delayed replication and specific interactions with the follicular dendritic cells of the spleen and bursa of Fabricius (68), where the vaccine virus stays sheltered before starting to replicate, coinciding with the decay in maternal antibodies (69).

Conclusion

The stability of IBDV in the environment and its resistance to common disinfectants explain the limitations of biosecurity in controlling the disease. This makes vaccination unavoidable, although its results have been inconsistent, in particular owing to the neutralisation of live vaccines by maternal antibodies and the antigenic and pathotypic variability of wild-type viruses.

While recombinant and immune-complex vaccines have enhanced the control of very virulent viruses and helped to minimise vaccination failure due to early vaccination, monitoring the evolution of antigenic variants is the best way to maximise the match between vaccine strains and circulating viruses.

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Table I

Characterisation of United States variant viruses by reactivity to different monoclonal antibodies (using antigen-capture enzyme-linked immunosorbent assay), according to Vakharia *et al.* (1994), with modifications (53)

MCA	Critical residues for MCA binding (5, 7, 30)	Classical virulent strains	Very virulent virus	Variant virus (USA)		
				E/DEL	GLS	DS326
B69	Q 249 (30)	+	+	–	–	–
R63	?	+	+	+	–	–
21	Q 253, D 279, A 284 (7)	–	+	–	–	–
8	?	+	+	+	+	+
10	G 318, D 323 (5)	+	+	–	+	+
179	E 311, Q 320 (30)	+	+	+	+	–
57	E 321 (30)	–	–	–	+	+
67	P or S 222, A 321 (5)	–	–	+	–	–
BK9	?	–	–	+	–	–

MCA: monoclonal antibody

\+ neutralisation present

– neutralisation absent

Table II

Reactivity of European, United States and African (Côte d'Ivoire) pathotypes to a battery of eight mouse monoclonal antibodies

Source: Domanska K. *et al* (41)

Virus strain	Origin	Pathotype	Reactivity to monoclonal antibodies (percentage)*							
			MCA 1	MCA 3	MCA 4	MCA 5	MCA 6	MCA 7	MCA 8	MCA 9
F52/70	Great Britain	Classical	72	71	58	59	88	96	91	76
Cu1 wt	Germany	Classical	68	135	106	58	116	107	133	50
D78	Netherlands	Attenuated	+	+	+	+	+	+	+	+
PBG98	Great Britain	Attenuated	+	+	+	nt	+	+	+	+
Var. E	United States of America	Variant	nt	2	nt	nt	6	nt	8	nt
Var. GLS	United States of America	Variant	nt	0	nt	nt	0	nt	0	nt
88180	Côte d'Ivoire	Very virulent	111	2	9	58	124	132	77	107
89163	France	Very virulent	58	1	6	27	95	86	76	73
91168	France	Very virulent	62	1	15	46	96	50	3	68
9448	Poland	Very virulent	65	1	8	39	109	112	84	76
Moh/96	Hungary	Very virulent	*78*	*5*	*17*	*82*	*152*	*171*	*136*	*49*

MCA: monoclonal antibody

* The higher the percentage, the higher the affinity of the strain for the monoclonal antibody

+ indicates the presence of neutralisation

nt: not tested

Table III

Critical residues for monoclonal antibody binding

Source: Eterradossi N. *et al.* (26)

Monoclonal antibody	Criticial residues for MCA binding
MCA 1	?
MCA 3	P 222 and G 223
MCA 4	P 222 and G 223
MCA 5	Q 249
MCA 6	Sequence 318–323
MCA 7	Sequence 318–323
MCA 8	Q 324
MCA 9	?

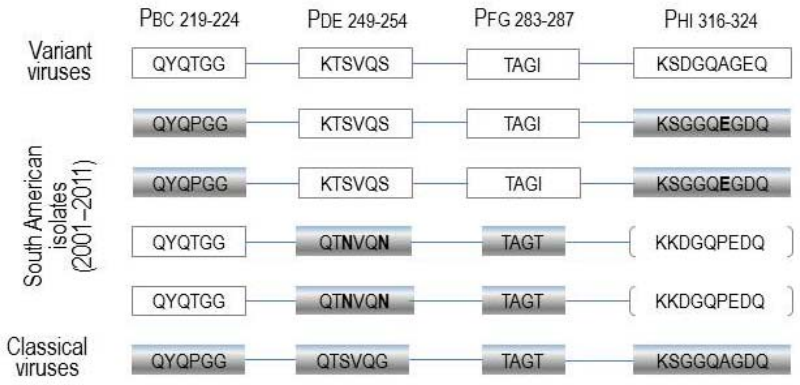

Fig. 1

South-American virus strains with natural recombination

Consensus sequences of amino acids in the PBC, PDE, PFG and PHI domains of the VP2 hypervariable region are shown in white rectangles for variant viruses (variants E/Del, V1 and F3) and in grey rectangles for classical viruses (strains F52- 70, Bursa vac, Cu-1, STC and 228E). Amino acids that do not conform to typical sequences of variant and classical viruses are shown in bold. Unique (atypical) amino acid sequences in the PHI domain are shown in parentheses. *Source*: Jackwood (21)

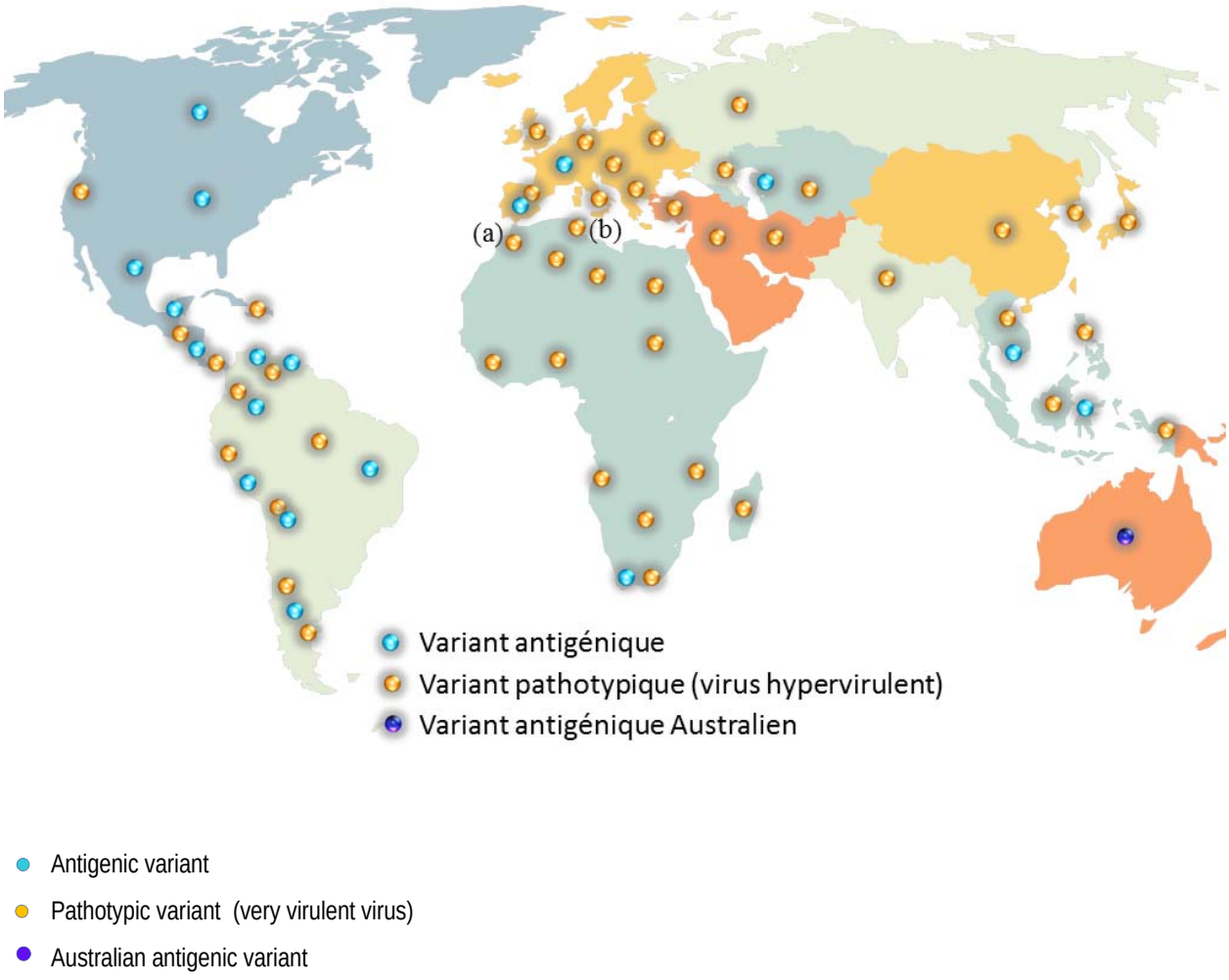

Fig. 2

Geographic distribution of the antigenic and pathotypic variants of infectious bursal disease virus according to Jackwood (personal communication, August 2013), with a few modifications on the basis of: *a)* Tahiri *et al.* (28); *b)* Mardassi *et al.* (29)

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                                        279┐      ┌284
D78      5′SVHGLVLGATIYLIGFDGTTVITRAVAANNGLTTGTDNLMPFNLVIPTNE 3′ 300
OKYMT      ..Q..T.............A..................I.........S.    300
IM         ..Q................A.........D....A................    300
EDGAR      ..Q..........................D....A................    300
STC        ..Q.........F..............D....A................    300
OKYM       ..Q..I.............A.........D....A....I.........S.    300
UK661      ..Q..I.............A.........D....A....I.........S.    300
HK46       ..Q..I.............A.........D....A....I.........S.    300
```

Fig. 3

Determination of the VP2 residues responsible for virulence by sequence alignment of the hypervariable domain of virulent strains (IM, EDGAR, STC, OKYM, UK661, HK46) and of cell-culture-adapted (OKYMT) strains with that of attenuated strain D78

A point under the letter indicates that the amino acid of the virus strain is the same as that found at the same position in strain D78. A letter under the letter indicates that the amino acid of the virus strain is different from that found at the same position in strain D78. *Source*: Brandt *et al*. (7)