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Running title: Detection of IBV Italy 02 in Morocco.

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Abstract

The aim of this study was to investigate the prevalence and diversity of infectious bronchitis virus (IBV) genotypes in poultry flocks in 16 areas of Morocco between 2010 and 2014. A total of 360 flocks suspected of being infected by IBV were screened for the IBV N gene using real time RT-PCR. Flocks were classified into four groups according to their IBV vaccination programme. Group 1 contained unvaccinated birds. Group 2, received a single application of live H120 vaccine. Groups 3 and 4 birds received one or two booster vaccination(s) respectively, mostly using the H120 vaccine. The real time RT-PCR results showed that 51.7% of the flocks were positive for the IBV genome with geographical disparities.

Molecular characterization of IBV was performed on 50 RT-PCR positive samples by partially sequencing the S1 gene, including the hypervariable regions (nucleotides 705-1097). Two predominant genotypes were detected, with the Massachusetts type dominating (66%), among which 25% of the samples were identical to the H120 vaccine. The second most common genotype (present in 32% of the flocks) was surprisingly Italy 02, revealing the first detection of this genotype in Morocco and also in Africa. 793B, the predominant genotype in the late 1990s in Morocco, was only detected in one occasion and was identical to the 4/91 vaccine strain.This study highlights the high prevalence of IBV in poultry farms in Morocco and confirms its continuous dynamic changes and evolution.

Introduction

Infectious bronchitis (IB) is an acute and highly contagious viral disease affecting chickens of all ages, causing respiratory distress, nephritis, reduction in egg production and quality and increasing mortality rate. The disease is probably endemic in all regions where poultry are reared intensively. The causative agent is infectious bronchitis virus (IBV) belonging to the Gammacoronaviruses within the *Coronaviridae* family (Cavanagh, 2007). IBV has a single-stranded, positive sense RNA genome (Boursnell *et al.,* 1987), which encodes three major structural proteins, the nucleocapsid protein (N), the membrane protein (M) and the spike glycoprotein (S) which is post-translationally cleaved into two subunits S1 and S2 (Cavanagh, 1983a, 1983b).

A large number of IBV serotypes and variants have been described worldwide (De Wit *et al.,* 2011). Many of them do not completely cross-protect (Gelb *et al.,* 1991; Cook *et al*., 2012). In North Africa, most of the information on IBV comes from Egypt, Morocco and Tunisia, where both indigenous and classical IBV variants appear to be present (De Wit *et al.,* 2011). In Western Africa, a high prevalence (26%) of IBV in poultry was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and a novel IBV variant 'IBADAN' has been described (Ducatez *et al.,* 2009).

In Morocco, the first isolation and characterization of IBV from poultry flocks was reported by El-Houadfi & Jones (1985). Five isolates designated D, E, F, H and M were related serologically to the Massachusetts (Mass) serotype, while a new genotype, G strain was reported to differ from the Mass serotype and others European serotypes known at that time. The authors demonstrated under experimental conditions that Mass vaccines provided poor protection against the G strain (EL Houadfi *et al.*, 1986). Interestingly, S1 sequence data

have shown that IBV G and 4/91 strains are very closely related, probably sharing a common origin (Jones *et al.*, 2004).

In 2004, a study investigated the relationships between IBV and outbreaks of nephropathogenic disease observed in broiler flocks in Morocco between 1996 and 2000 (Alarabi, 2004). Three different groups of IBV strains were identified using RT-PCR coupled with restriction fragment length polymorphism (RFLP) and isolates were classified into 3 groups. Isolates of group I were identified as Mass, the other IBV groups (group II and group III) were different from Mass types. The isolate 12/97, representative of group III, was found to cause severe kidney lesions and high mortality. Cross protection studies have shown that dual vaccination using the H120 and 4/91 vaccines at 1 day-old and 14 days of age respectively provided better protection against isolate 12/97 in comparison to single vaccination with H120 or 4/91. In addition, this strain was very closely related to the Moroccan G strain (Alarabi, 2004; EL Houadfi *et al.*, 2004). El Bouqdaoui *et al.* (2005), while studying nephropathogenic IBV, identified five genotypes, three of which differed from vaccine strains, using RT-PCR and RFLP techniques.

Since 2005, several IBV outbreaks have been suspected to be present in poultry flocks in different regions of Morocco, despite the use of H120 and 4/91 vaccines (EL Houadfi, personal communication).The first goal of the present study was to investigate the prevalence of IBV infection in Moroccan poultry flocks, in various geographic regions over a four year period (2010-2014) where different vaccination strategies had been applied. The second objective was to identify the IBV genotypes circulating in the country and their importance and frequency in the field.

Materials and Methods

Sampling scheme. Sixteen distinct Moroccan geographical areas were selected for this study (Table 1). A sampling scheme based on Probability Proportional to Size (PPS Sampling) was adopted. To take into account the possible importance of the larger units in the poultry flocks population, regions were selected with the probability proportional to geographical area. The following formula was used to calculate the sample size for estimating a given proportion with a 5% error margin (e):

$$n = \frac{(1{,}96)^2 p(1-p)}{e^2 + \frac{(1{,}96)^2 p\,(1-p)}{N}}$$

n = Sampled units (sample size = 360)

p = theoretical proportion (about 44%)

e = 5% error margin (95% confidence level)

N = Existing broiler production units (Population size = 6407)

A total of 360 flocks (328 broilers, 21 layers and 11 breeders) were sampled and their origins are shown in Table 1. All chickens experienced one or more of the following clinical signs or lesions: respiratory distress, nephritis, tracheitis, airsacculitis and, in the case of layers or breeders, drops in egg production and poor egg quality. Information on sampled cases related to age, mortality, clinical symptoms and *postmortem* lesions were recorded.

Vaccination protocols were given particular attention, especially day of vaccination, number of vaccinations and vaccine strains used. Indeed in Morocco, vaccine strains commercially available are of the Mass type (H120, Ma5 and modified Massachusetts strains), 793B type (4/91 and CR88) and since 2013 Arkansas type. However, because of its low price, H120 is the most common used vaccine in broiler chickens.

Treatment of samples. Tracheal swabs, kidney, trachea and lung tissues were collected from flocks suspected of being infected by IBV. They were immediately placed on ice in sterile transport minimum essential medium (MEM) containing 5% antibiotics (20,000IU/ml penicillin, 10,000 µg/ml streptomycin, and 5000 µg/ml kanamycin) and sent to BIOPHARMA laboratory where they were immediately stored at -80°C until analyzed. After thawing, each sample was homogenized with Dulbecco's modified Eagle's medium (DMEM). Tissue suspensions were centrifuged at 8000g for 20 min at 4°C. Then, 500µl of supernatant was clarified at 12000g for 1 min and immediately processed.

RNA Extraction. Five samples from each case were pooled. Viral RNA was extracted from 50µl of the field specimens using the MagMaxTM-96 AI/ND viral RNA isolation kit (Applied Biosystem/Ambion, Austin TX, USA) according to the manufacturer's instructions. Each sample of extracted RNA was recovered from the plate and subjected to reverse transcriptase RT-PCR amplification.

One step real time RT-PCR for N gene detection. The oligonucleotides and the probe described by Rosie *et al*. (2010) are located in the conserved N gene, at nucleotide position 741–1077 of the Mass H120 strain (GenBank accession no. AM260960). Negative controls (H_2O) and Newcastle Disease Virus, (NDV strain HB1) were included. A one step RT-PCR was carried out using Invitrogen kit (SuperScript® III Platinum, Life Technologies, USA). The reaction volume contained 12.5µl 2× RT-PCR buffer mix, 0.5µl $MgSO_4$ (50mM), 0.5µl Rox (25mM), 4.75µl nuclease free water, 0.5µlM-MULV reverse transcriptase enzyme (200 U), 0.5µl primers to a final concentration of 10 µM, 0.25µl probe to a final concentration of 10 µM and 5µl RNA template. The reaction was carried out in StepOneTM Plus real-time PCR system (Smart cycler Cepheid, USA) at 50°C for 15 min, 95°C for 5 min, and 40 cycles

of 95°C for 15s and 60°C for 45s. Amplifications were recorded, analyzed, and the threshold cycle (Ct) determined with the StepOne software (Smart Cycler).

Nested RT-PCR for S1 gene amplification. Fifty samples positive by RT-PCR were selected for further study in order to include all epidemiological criteria: vaccination status, severity of clinical signs, years of detection, and to cover all regions of Morocco. However, all 6 positive samples of group 4 (3 times vaccinated) were sequenced (Table 1), in order to understand better the vaccine failures. The RT-PCR products were subjected to S1 gene nested RT-PCR amplification, using the primers and protocol described by Jones *et al*. (2005) and Worthington *et al.* (2008).

DNA sequencing. Fifty selected RT-PCR products (393bp) generated with the nested RT-PCR and containing a region known to vary between strains were purified using the Gene Clean Kit (ExoSAP-IT), according to the manufacturer's recommendations and sequenced using the primers (SX3+ and SX4-). Determination of the nucleotide sequences was performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit and the second purification was performed using the Big Dye X terminator Purification Kit.

Nucleotide sequence analyses. Assembly and analysis of sequence data were conducted using the BioEdit Software version 5.0.9 (Hall, 1999).Phylogenetic analysis and tree construction for the S1 glycoprotein were generated using the maximum likelihood (ML) method with 1,000 bootstrap replicates with MEGA software Version 5.05 program (Tamura *et al.,* 2011), and bootstrap values above 50 were labeled on major tree branches for reference.

As the primers used in this study do not differentiate between vaccine and field viruses of the same genotype, we compared the sequences of the viruses detected as Mass and 793B genotypes with those of the vaccine types published in NCBI (H120: accession number M21970 ; 4/91: accession number AF093793).

Statistical analyses. Statistical Package for the Social Sciences (SPSS) version 13 was used for statistical analyses. Descriptive statistics including percentages, means and frequency distribution, were calculated for each of the variables. The Chi-square test was used to investigate the correlations of IBV detection between regions and between groups. The frequency of IBV infection in different groups was calculated using logistic regression analysis. A *p*-value of less than 0.05 was considered significantly different.

Genbank accession numbers. The partial S1 gene sequences of the viruses analyzed in this study were deposited in GenBank with accession numbers shown in Table 2.

Nucleotide and amino acid deduced S1 sequences of reference IBV strains included in the comparisons were available in GenBank under the following accession numbers: Ark99 (M99482); 4/91 (AF093793); Beaudette (X02342); M41 (X04722); H120 (M21970); Ma5 (AY561713); D1466 (M21971); Moroccan-G/83 (EU914938); QX (AF193423); D274 (X15832); NGA/A116E7/2006 (FN182257); Q1 (AF286302); Gray (L14069); Spain/00/336 (DQ386098); Spain/99/327 (DQ386097); Spain/05/866 (DQ386102); Spain/04/221 (DQ386103); Italy-02 (AJ457137); UK L-633/04 (DQ901376); Spain/98/315 (DQ386095); Spain/92/35 (DQ386091); Spain/95/193 (DQ386093); Spain/04/22(DQ386100).

Results

Field IBV vaccination. In this study, several different vaccination protocols were recorded (Table 1). 87 flocks out of 360 (24.2%) did not receive any IBV vaccine (group I). 205 flocks (56.9%) received one vaccination with H120 between one and seven days of age (group 2). 38 flocks (10.6%) were vaccinated twice, at one week of age with H120 followed by a booster vaccination between two and three weeks of age (group 3). 30 flocks (8.3%) received three vaccinations at 1, 2, and 3 weeks of age (group 4). Among the 263 vaccinated flocks studied, only 13 flocks had been boosted with IB 4/91 vaccine. The remaining flocks were boosted with H120.

Virus prevalence in different vaccination status. The number of selected farms based on the statistical (SPSS) sampling method in southern, western, eastern and central regions of Morocco is shown in Table 1. Among 360 flocks sampled, 273 had been vaccinated against IBV (75.8%). IBV detections occurred in broiler flocks aged between 19 and 45 days and in commercial layer flocks aged between 17 and 51 weeks, distributed in all areas of Morocco. Among these flocks, 62.5% showed respiratory signs.

The real time RT-PCR results revealed that 51.7% (186/360) of the suspected infected IBV flocks were indeed IBV-positive. IBV was detected in all regions. In group 1 (unvaccinated birds), the relative prevalence of IBV infection reached 66.7%, followed by 54% in group 2 (1 vaccination), 21% in group 3 (2 vaccinations) and 20% in group 4 (3 vaccinations).The effect of vaccination in different groups was investigated. The odd ratios obtained between group 1 and group 2 was 1.257 (95% CI, 0.826-1.913), with no statistically significant effect of vaccination (*p*: 0.286). In groups 3 and 4, the risk of infection with IBV was reduced by 75% and 80%, respectively, and the odd ratios were 0.281 (95% CI, 0.123-

0.644, (p 0.003) and 0.208 (95% CI, 0.083-0.523, p<0.000001). Despite the difference of IBV prevalence between regions, no statistically significant differences were found.

IBV genotypes. Among the 186 samples positive for IBV by real time RT-PCR (51.7%), 50 samples were sequenced for the S1 gene using both primers (SX3+ and SX4-) described previously by Jones *et al.* (2005). Partial nucleotide sequences of the S1 genes (393 bp) of the Moroccan IB viruses were compared with published IBV sequences using BLAST search within the EMBL/GenBank database (Table 2). During the four years of this study, the predominant genotype detected was Mass (66%), followed by Italy02 (32%) and only one sample belonged to the 793B genotype (2%). Among the two genotypes, the Mass genotype was dominant between 2011 and 2013, while Italy 02 was constantly present during the four years of the study.

In unvaccinated flocks both Italy 02 and Mass wild type (differentiated from H120 vaccine by nucleotide alignment) were found to be more or less equally distributed (found at prevalence of 55% and 45%, respectively). In vaccinated flocks, Mass wild type (Wt) and Italy 02 were detected in all groups (2, 3, and 4) but with different frequencies: Mass Wt clearly dominated in groups 2 and 4 (Figure 1).

The distribution of the IBV genotypes between regions demonstrated that the Mass genotype is ubiquitous and was found in almost all regions. However Italy 02 was concentrated more in the centre and north regions of Morocco (Figure 2).The amino acid sequences of the S1 gene of the 50 IBVs in different regions and different years, compared to the reference strains are represented in Figure 3. Phylogenetic analyses confirmed that Moroccan viruses are grouped into three genotypes: Mass, Italy 02 and 793B. The Moroccan IBV field viruses had amino acid sequence similarity variation of 42.4% between IBV/Morocco/23/2013 and IBV/Morocco/16/2013 and 100% between IBV/Morocco/39/2010

and IBV/Morocco/05/2010. In comparison with 23 reference strains, they had amino acid sequence similarity variation between 44.4% (IBV/Morocco/27/2012 and D1466) and 98.4% (between IBV/Morocco/05/2010 and H120) (data not shown).

Nucleotide sequence alignments and comparison revealed that 25% of the field viruses had 100% sequence identity with the commercial H120 live vaccine (IBV/43/2013 to IBV/28/2012), while 75% were different from the vaccine strain and grouped in 2 clusters (Figure 3). The first and main cluster (with 96% of the non-vaccine Mass viruses) was detected in all regions of the country (IBV/40/2013 to IBV/11/2012), while the second cluster was observed only in the southern regions of Morocco and included IBV/48/2013 and IBV/23/2013, which were detected in 2013 (Figure 3). In this study, the 793B genotype, a virus associated with nephritis, which predominated in the 1980s and 1990s in Morocco (Jones & EL Houadfi, 1985, Alarabi, 2004; El Bouqdaoui *et al.,* 2005) was detected only on one occasion. This virus was found to share 100% nucleotide identity to the 4/91 vaccine strain (accession number AJ457137).

Discussion

In this study, a comprehensive survey of IBV throughout Morocco was performed on 360 flocks showing clinical signs of respiratory infection, kidney diseases, or, in layers and breeders, loss of egg production and quality. IBV infection was found to be a common disease in poultry farms and occurred in almost all regions. IBV RNA was detected in 51.7% of the flocks tested. This prevalence is relatively similar (59%) to that found in Western Europe (Worthington *et al.,* 2008). This high frequency found in Morocco and in Western Europe might be due to the fact that flocks sampled were experiencing clinical signs and suspected to be infected with IBV. However in Morocco, many factors such as the lack of an

appropriate vaccination programme and application, or lack of biosecurity, for example in multi-age rearing systems which are still wildly used. The differences in IBV prevalence found among groups 2, 3, and 4, illustrated that the more often birds were vaccinated the fewer were infected. Groups 3 and 4, which received respectively one and two booster vaccinations, were better protected than flocks receiving a single vaccination. Similar findings have been reported by Jackwood *et al*. (2009). Those authors found that two vaccinations against IBV, a prime at 1 day of age and a booster between 14–18 days of age, were necessary to induce a protective local antibody response in the upper respiratory tract. Ignjatovic & Galli (1995) also reported that chicks vaccinated at 14 days of age had significantly higher titres of S1 and S2 antibodies than chicks vaccinated at either 1 or 7 days of age. Controlling IB only by increasing the frequency of vaccination does not always work, especially when field strains are antigenically distinct from the applied vaccines strains. This situation is well illustrated in this study, which shows that IBV RNA was detected in 20% of the flocks in group 4, despite the use of three applications of H120 vaccine. This suggests that other genotypes, such as Italy 02 genotype (IBV09 and IBV38 specimens from group 4), or probably wild type Mass genotype (IBV11, 35, 36 and 39 from group 4) [Table 2] are circulating in Morocco, as has been shown previously (El Houadfi *et al.,* 1986; Alarabi, 2004; El Bouqdaoui *et al.,* 2005). In North Africa, IBV variants have been poorly studied. However, Bourogâa *et al*. (2009b) identified new IBV isolates for the first time in Tunisia, which co-circulated along with the Mass type, causing severe clinical diseases and high economic losses to the poultry industry (Bourogâa *et al*., 2009a, 2009b). In Egypt, isolates related to Mass, D3128, D274, D-08880, 4/91 and the novel genotype; Egypt/Beni-Suef/01 were characterized (Abdel-Moneim *et al*., 2006; Mahmood *et al.,* 2011). Indeed, new strains/genotypes of IBV are in continuous emergence in Egypt (Abdel-Moneim, 2012).

During the last decade, two new economically important field types of IBV named QX and Italy 02 genotypes, were isolated in domestic poultry in Europe (Jones *et al.*, 2005; Bochkov *et al*., 2007; Dolz *et al*., 2007; Valastro *et al*., 2010; Krapez *et al*., 2011; Ganapathy *et al*., 2012), China (Beato *et al*., 2005; Zhao *et al*., 2014) and in the Middle East (Amin *et al*., 2012). However, in Africa only QX viruses have so far been detected, in Zimbabwe (Toffan *et al*., 2011).

The survey of IBV genotypes in Western Europe using universal primers for IBV, followed by sequencing of positive PCR products for the S1 spike gene, revealed that 793B was the most common type detected, followed by Mass, including different vaccine strains. That study revealed the emergence of Italy 02 genotypes in all countries from which samples were received, whereas QX was the fourth most common genotype, predominant in all countries except the United Kingdom and Spain (Worthington *et al*., 2008).

Variation in the IBV S1 gene is very often used to distinguish between different IBV genotypes. The generation of genetic variants is thought to be the result of very few amino acid changes in the spike (S) glycoprotein of IBV (Kant *et al*., 1992; Cavanagh *et al*., 1992; Wang, 2000; Dolz *et al*., 2006). The S1 primer set was used previously for characterizing field samples in Western Europe and was shown to detect a wide range of IBV genotypes (Jones *et al*., 2005; Worthington *et al*., 2008), which brings confidence in our results, although the possibility of strains harbouring mismatches at primer locations cannot be ruled out.

The results of our study show that the most frequently detected IBV genotypes were Mass and for the first time in Africa, Italy 02 type. The Mass genotype was predominant with a prevalence of 66%.This result is not surprising as Mass vaccines have been used in Morocco since 1960. Previous results published in Morocco showed that the Mass IBV type was the first isolated in Morocco in the 1980s (EL Houadfi *et al*., 1986). The authors then

characterized six isolates of IBV, among which five isolates were related serologically to the Mass serotype. The analysis of the nucleotide sequences of the Mass genotype over the S1 section of the spike gene indicated that 25% of the Mass viruses were identical to live H120 vaccine. Again, this was expected considering the extensive use of H120 vaccines. Cavanagh *et al*. (1999) reported that when Mass type IB vaccines were applied at 1 day-old in the hatchery, vaccine virus could later be detected in all broiler flocks tested by RT-PCR using tracheal swabs, with maximal amounts during the first week of life. Naqi *et al*. (2003) using RT-PCR and sequencing, also demonstrated the persistence of vaccine virus for many weeks after administration. Therefore, it was not surprising that we detected vaccine strains in this survey.

The remaining 75% of detected Mass viruses indicated that wt Mass viruses are widespread in Morocco and might cause significant losses to the poultry industry. Further investigations related to cross protection and pathogenicity of the two Mass variants detected in the country are needed, especially as Callison *et al*. (2001) have demonstrated that nucleotide sequences of the whole S1 spike gene of the 4/91 vaccine strain and pathogenic virus of 793B only differ by 0.6%. In this way we could perhaps determine whether we indeed detect wild Mass viruses or whether they might have been vaccine-derived variants.

The second most frequently detected genotype was Italy 02. This is the first study in which Italy 02 types has been detected in Morocco and in Africa. The geographical proximity and close transboundary of Morocco and Europe may explain in part the dominance of this genotype in the north and central regions of Morocco. Spain is also the only country exporting breeder chicks to Morocco (ONSSA; 2014). The Italy 02 genotype was originally isolated in Italy in 1999 (Bochkov *et al*., 2007) and it has been reported in Spain since 1997 (Dolz *et al*., 2006). Dolz *et al*. (2008, 2012) investigated IBV variants in Spain and reported that Italy 02 is the predominant genotype in that country. This genotype was detected

throughout Western Europe (Worthington *et al*., 2008) and in Russia (Bochkov *et al*., 2006). Recently, Dolz et al., (2014) reported that Italy 02 prevalence in Western European countries decreased shortly after its emergence, however, Spain was the only country where this genotype was predominant up to 2011, before being replaced by QX in 2012 and by 4/91 since 2013 (Dolz *et al*., 2014). In Morocco, the Italy 02 genotype was detected for the first time in 2010 and its frequency seemed to increase so that it become the most common IBV genotype in 2014. A retrospective study on Moroccan isolates collected since 1982 is ongoing to determine when Italy 02 was introduced to Morocco.

Before starting this study we were expecting to detect the QX genotype because of its widespread distribution in many countries, but the sequencing result of 50 IBV did not show any QX-like genotype. Interestingly, it was surprising to find that the 793B genotype had almost disappeared from the country, while it was predominant in the 1980s and 1990s (El Houadfi *et al*., 1986; Alarabi, 2004; El Bouqdaoui *et al*., 2005). It would thus be worth trying to understand what might be the main cause of 793B decline in Morocco. This could probably be related to the intensive use of 4/91 vaccine for preventing the outbreaks of renal disease, which have affected the country since 1995, and against which it has been found that 4/91 was very protective in combination with H120 (Alarabi, 2004; EL Houadfi *et al*., 2004). In Spain, Dolz *et al*. (2008) suggested that the emergence of Italy 02 displaced 793B genotype, which is also possibly the case in Morocco. This phenomenon illustrates the continuous evolution and the dynamic changes of IBV, which complicate the establishment of vaccine strategies to control it. These strategies need to be adapted continuously to the field situation in Morocco.

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Figure 1. *Distribution of IBV genotypes detected in flocks in which IB H120 vaccine was applied once (V1), twice (V2) or three times (V3).*

Mass Wt: Wild type of Massachusetts genotype; Mass V: Vaccine strain of Massachusetts genotype.

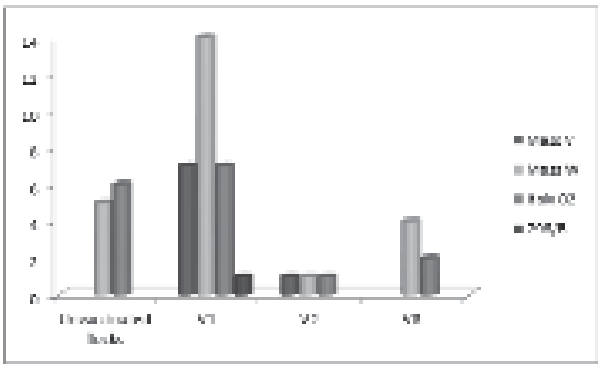

Figure 2. *Map showing the distribution of IBV genotypes and the region of Morocco where they were detected.*

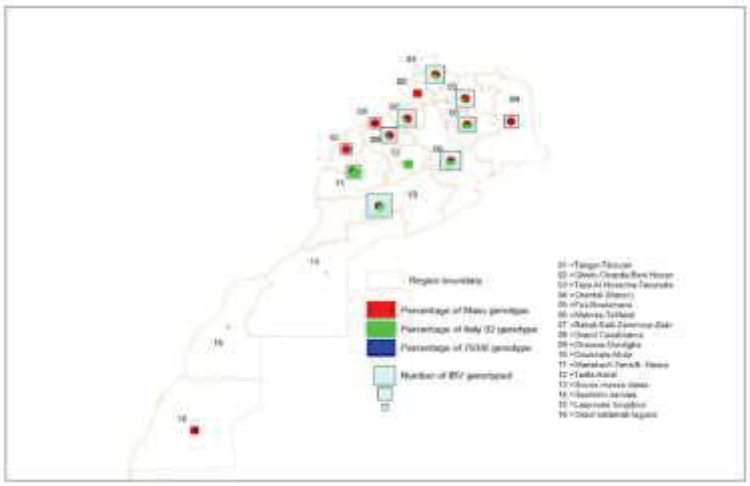

Figure 3. *Phylogenetic tree (Maximum Likewood tree) of the S1 gene from 50 IBV viruses.*

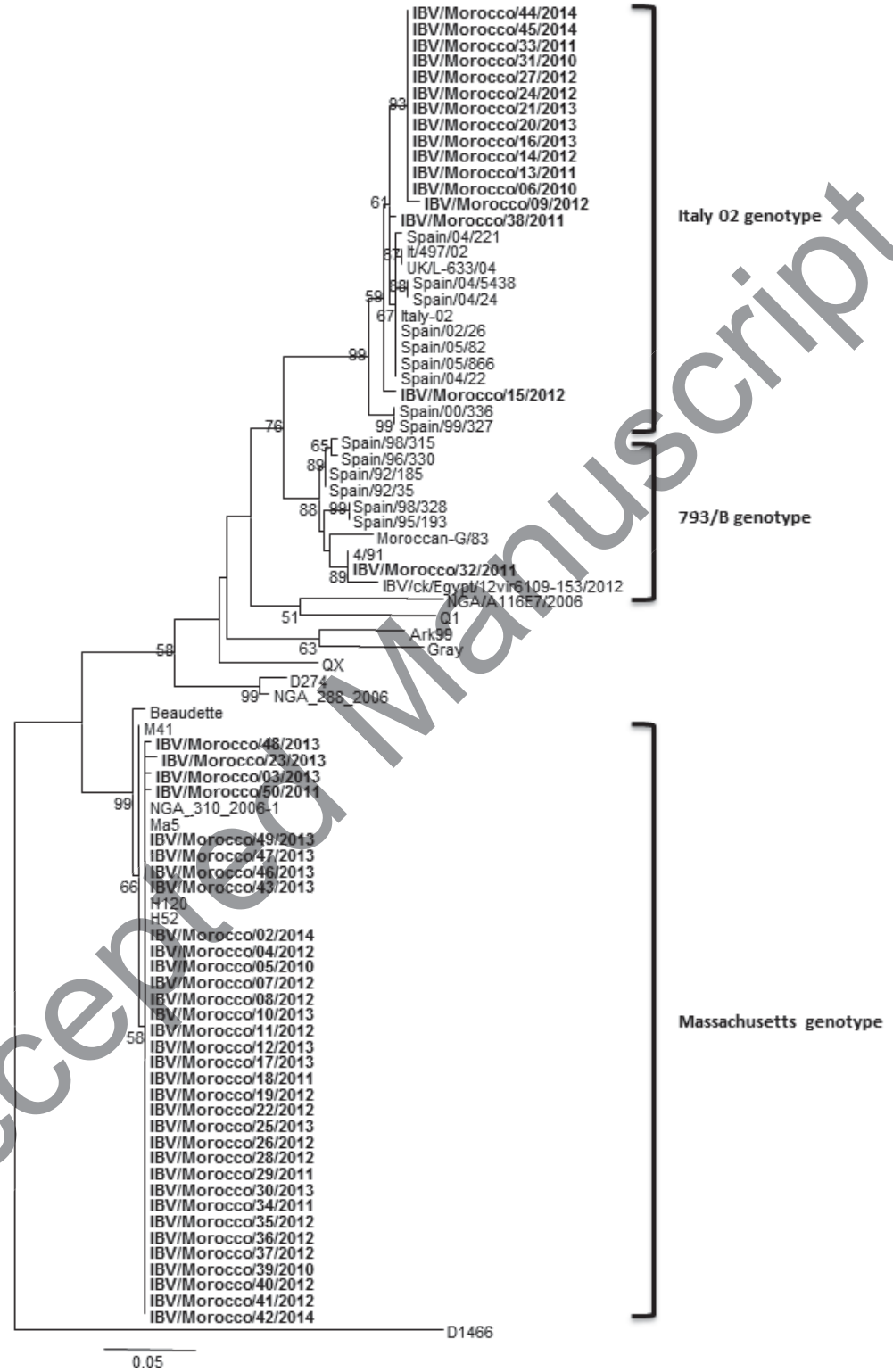

Table 1. *Prevalence of Infectious Bronchitis infection in poultry chickens from 16 regions of Morocco.*

Region	Location[a]	Unvaccinated flocks	Vaccinated flocks			Total
		Group 1[b]	Group 2[c]	Group 3[d]	Group 4[e]	
Fès-Boulemane	N	2/4 (50)[f]	8/16 (50)	1/1 (100)	0/3 (0)	11/24 (45.8)
Casablanca	W	0	6/8 (75)	0/1 (0)	0	6/9 (66.7)
Marrakech-Tensift- Haouz	C	0/2 (0)	4/12 (33.3)	4/13 (30.7)	0/9 (0)	8/36 (22.2)
Rabat-Salé-Zemmour-Zaër	NW	1/1 (100)	14/22 (63.6)	0/2 (0)	1/1 (100)	15/26 (57.7)
Doukkala-Abda	WC	7/10 (70)	6/13 (46)	0/12 (0)	0/4 (0)	13/39 (33.3)
Meknès-Tafilalet	NC	7/12 (58)	11/19 (57)	1/1 (100)	1/3 (33.3)	20/35 (57.1)
Tanger-Tétouan	NW	5/7 (71.4)	7/15 (46.6)	0	0/2 (0)	12/24 (50)
Tadla-Azilal	C	1/1 (100)	5/12 (41.6)	1/3 (33.3)	3/5 (60)	10/21 (47.6)
Gharb-Chrarda-Ben Hssen	NW	1/3 (33.3)	3/11 (27.2)	0/1 (0)	0	4/15 (26.7)
Oriental	E	11/15 (73.3)	2/4 (50)	0	0	13/19 (68.4)
Taza-Al Hoceima-Taounat	N	8/10 (80)	1/8 (12.5)	0	0	9/18 (50)
Chaouia-Ourdigha	NC	7/9 (77.7)	11/23 (47.8)	1/4 (25)	0	19/36 (54.3)
Laâyoune-Boujdour	S	0	0/1 (0)	0	1/3 (33.3)	1/4 (25)
Souss massa Daraa	C	8/13 (61.5)	37/41 (90.2)	0	0	45/54 (81.8)
Guelmim Smara	NS	0	0	0	0	0
Oued Ed-Dahab-Lagouir	S	0	0	0	0	0
Total		58/87 (66.7%)	114/205 (54%)	8/38 (21%)	6/30 (20%)	186/360 (51.7%)

[a] Location of the regions in Morocco, N: Northern, W: Western, S: Southern, C: Central, E: Eastern.
[b] Group 1 contained unvaccinated flocks.
[c] Group 2 one application of Mass H120 live vaccine.
[d] Group 3 two applications of Mass H120 live vaccine.
[e] Group 4 three applications of Mass H120 live vaccine.
[f] Number positive/ number examined (%positive).

Table 2. *Characterization of Moroccan infectious bronchitis viruses from poultry flocks between 2010 and 2014.*

Isolate number[a]	Region of Morocco	Age[b]	Major clinical signs	Vaccination status	Genotype	GenBank accession numbers
IBV/Morocco/01/2011	Taza-alhoceima	20 d	Respiratory	NV[c]	Italy02	KM594187
IBV/Morocco/02/2014	Fès-Boulemane	24 d	Respiratory	1V[d]	Mass	KM594215
IBV/Morocco/03/2013	Casablanca	26 d	Respiratory	2V	Mass	KM594216
IBV/Morocco/04/2012	MarrakechTensift	34 d	Respiratory	1V	Mass	KM594217
IBV/Morocco/05/2010	Rabat-Salé	27 d	Respiratory	1V	Mass	KM594218
IBV/Morocco/06/2010	Meknès-Tafilalet	30 d	Respiratory	1V	Italy02	KM594219
IBV/Morocco/07/2012	Rabat-Salé	33 d	Respiratory	1V	Mass	KM594220
IBV/Morocco/08/2012	Doukkala-Abda	28 d	Nephritis	1V	Mass	KM594221
IBV/Morocco/09/2012	Meknès-Tafilalet	40 w	Respiratory	3V	Italy02	KM594206
IBV/Morocco/10/2013	Meknès-Tafilalet	32 d	Respiratory	1V	Mass	KM594222
IBV/Morocco/11/2012	Laâyoune-Bouj	17 w	Respiratory	3V	Mass	KM594223
IBV/Morocco/12/2013	Meknès-Tafilalet	31 d	Respiratory	1V	Mass	KM594224
IBV/Morocco/13/2011	Fès-Boulemane	41 d	Nephritis	NV	Italy02	KM594203
IBV/Morocco/14/2012	Meknès-Tafilalet	32 d	Nephritis	NV	Italy02	KM594195
IBV/Morocco/15/2012	Tanger-Tétouan	42 d	Respiratory	NV	Italy02	KM594201
IBV/Morocco/16/2013	Fès-Boulemane	37 d	Respiratory	1V	Italy02	KM594204
IBV/Morocco/17/2013	Oriental	30 d	Respiratory	NV	Mass	KM594225
IBV/Morocco/18/2011	Marrakech-Tensi	25 d	Respiratory	2V	Mass	KM594226
IBV/Morocco/19/2012	Casablanca	32 d	Respiratory	1V	Mass	KM594191
IBV/Morocco/20/2013	Marrakech-Tensi	32 d	Nephritis	1V	Italy02	KM594207
IBV/Morocco/21/2013	Souss massa Dar	23 d	Respiratory	NV	Italy02	KM594193
IBV/Morocco/22/2011	Rabat-Salé	28 d	Respiratory	1V	Mass	KM594227
IBV/Morocco/23/2013	Doukkala-Abda	30 d	Respiratory	1V	Mass	KM594228
IBV/Morocco/24/2012	Tanger-Tétouan	31 d	Nephritis	NV	Italy02	KM594196
IBV/Morocco/25/2013	Meknès-Tafilalet	42 d	Respiratory	1V	Mass	KM594229
IBV/Morocco/26/2012	Tanger-Tétouan	36 d	Respiratory	1V	Mass	KM594230
IBV/Morocco/27/2012	Tadla-Azilal	31 d	Respiratory	1V	Italy02	KM594199
IBV/Morocco/28/2012	Gharb-Chrarda	36 d	Respiratory	1V	Mass	KM594231
IBV/Morocco/29/2011	Oriental	33 d	Respiratory	1V	Mass	KM594232
IBV/Morocco/30/2013	Chaouia-Ourdigh	30 d	Respiratory	1V	Mass	KM594233

IBV/Morocco/31/2010	Souss massa Dar	19 d	Respiratory	1V	Italy02	KM594205
IBV/Morocco/32/2011	Souss massa Dar	42 d	Nephritis	1V	793/B	KM594234
IBV/Morocco/33/2011	Souss massa Dar	28 d	Nephritis	1V	Italy02	KM594190
IBV/Morocco/34/2011	Chaouia-Ourdig	36 w	Respiratory	N/A[e]	Mass	KM594235
IBV/Morocco/35/2012	Taza-Al Hoceima	29 d	Respiratory	3V	Mass	KM594236
IBV/Morocco/36/2012	Taza-Al Hoceima	20 w	Respiratory	3V	Mass	KM594237
IBV/Morocco/37/2012	Fès-Boulemane	27 d	Respiratory	1V	Mass	KM594238
IBV/Morocco/38/2011	Souss massa Dar	51 w	Respiratory	3V	Italy02	KM594239
IBV/Morocco/39/2010	Taza-Al Hoceima	42 w	Respiratory	3V	Mass	KM594240
IBV/Morocco/40/2012	Tanger-Tétouan	35 d	Respiratory	1V	Mass	KM594241
IBV/Morocco/41/2012	Tanger-Tétouan	26 d	Respiratory	1V	Mass	KM594242
IBV/Morocco/42/2012	Souss massa Dar	42 d	Respiratory	1V	Mass	KM594243
IBV/Morocco/43/2013	Taza-Al Hoceima	34 d	Respiratory	NV	Mass	KM594244
IBV/Morocco/44/2014	Rabat-Salé	28 d	Respiratory	1V	Italy02	KM594188
IBV/Morocco/45/2014	Chaouia-Ourdigh	22 d	Nephritis	2V	Italy02	KM594189
IBV/Morocco/46/2013	Chaouia-Ourdigh	19 d	Respiratory	NV	Mass	KM594245
IBV/Morocco/47/2013	Oriental	21 d	Respiratory	NV	Mass	KM594246
IBV/Morocco/48/2013	Souss massa Dar	41 d	Respiratory	NV	Mass	KM594247
IBV/Morocco/49/2013	Souss massa Dar	33 d	Respiratory	1V	Mass	KM594248
IBV/Morocco/50/2011	Rabat-Salé	42 d	Respiratory	1V	Mass	KM594249

[a] The last value indicates the year of detection.
[b] d= days of age; w = week of age.
[c] NV= not vaccinated against IB.
[d] Vaccinated with IBV H120 vaccine once, twice, or 3 times.
[e] N/A= not known.