

Infectious bronchitis virus variants in chickens: evolution, surveillance, control and prevention

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ABSTRACT. Infectious bronchitis is a disease of the upper respiratory tract of chickens caused by a Gammacoronavirus (infectious bronchitis virus, IBV). Severe economic losses are caused by IBV due to a reduction in egg production and/or egg quality in layers in addition to poor feed conversion and increased condemnations in broiler chickens. The extreme variability of this virus is in part due to its RNA genome, which predisposes it to mutations and generates genetic variation. In addition, recombination events add to the variability of this virus. IBV variability was first described in 1956 by Jungherr. Since then, dozens of serotypes and hundreds of genotypes have been reported. Variant IBV strains are those that, can escape from the immunity generated by conventional strains, despite not being fully different from conventional strains affecting a geographic region. At the genomic level, these differences can be equal or greater than 5% of the hypervariable region of the S1 gene. These variant strains are usually restricted to geographic regions and most of the time are transient, reason why diagnostics and epidemiological surveillance are crucial to determine their existence and persistence. The main goal of surveillance is to assist the development of efficient preventative measures in the field. This review aims to critically analyse the literature related to IBV variability and judiciously comment and discuss on how to better prevent this poultry endemic disease.

Key words: IBV, variants, variability, evolution, surveillance.

INTRODUCTION

Understanding factors that might play a role in the generation of infectious bronchitis variants is crucial in order to perform surveillance, prevent their occurrence and establish prevention strategies. These factors involve inherent viral characteristics, management, vaccines and vaccination, immunity and concomitant infectious diseases. The purpose of this review is to merge research findings with clinical observations and laboratory expertise on the generation, diagnostic, surveillance and prevention of infectious bronchitis variant strains in commercial chicken production.

The infectious bronchitis coronavirus (IBV) is the causative agent of one of the most economically important diseases in modern poultry production, infectious bronchitis (IB), in chickens. The disease was first described by Schalk and Hawn in 1931. Nowadays the disease is endemic in most of the countries that possess a developed commercial poultry industry. Its global economic impact has been estimated as the second most damaging poultry disease after highly pathogenic avian influenza (TAFS-Forum 2011). The clinical picture is associated with upper respiratory tract infection characterised by conjunctivitis, tracheitis and loss of ciliary movement in the trachea. In addition, reproductive effects such as drop in egg production and alterations in the egg internal and

external quality can be seen in layer and breeder birds. If layer chicks get exposed early in their lives the virus can infect the oviduct, altering its normal development and inducing false layers (Broadfoot *et al* 1956, Crinion and Hofstad 1972, Gallardo *et al* 2019). Some IBV strains have kidney tropism inducing nephritis and urate deposition (Winterfield and Albassam 1984, Albassam *et al* 1986) while others have been associated to enteric disorders (El-Houadfi *et al* 1986, Hauck *et al* 2016). Secondary infections are common in meat-type birds affected by IBV inducing airsacculitis and increasing condemnations at the processing plant.

INFECTIOUS BRONCHITIS VIRUS

IBV is a single-stranded, positive sense RNA virus of the *Coronaviridae* family, genus *Gammacoronavirus*¹. The viral genome comprises two untranslated regions (UTR's) in its 5' and 3' ends, two overlapping reading frames (ORF's) encoding the structural polyproteins 1a and 1ab, and the region encoding the main structural proteins i.e. spike (S), envelope (E), membrane (M) and nucleocapsid (N). Finally, two accessory genes ORF 3 and ORF 5 encode for proteins 3a, 3b and 5a and 5b (Ziebuhr *et al* 2000). The S protein is located on the surface of the virus external membrane. During binding to the host cell, the S protein is cleaved on a cleavage site rich in basic bases into the amino-terminal S1 (~535 amino acids) and the carboxy-terminal S2 (~627 amino acids) (Cavanagh *et al* 1986). The S1 portion of the S protein of IBV is responsible for viral attachment to host cells, virus variability and eliciting neutralizing antibodies in

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¹ <http://www.talk.ictvonline.org/taxonomy/>

chickens (Cavanagh 1983, Cavanagh and Davis 1986, Kusters *et al* 1987, Casais *et al* 2003). The S1 displays the most genetic and phenotypic variability among different IBV strains (Cavanagh 1983, Cavanagh and Davis 1986, Kusters *et al* 1987) and is therefore the best target to assess variability. Variant IBV strains are those that, despite not being fully genetically different from conventional strains affecting a geographic region, are phenotypically different and escape from the immunity generated by conventional serotype-specific vaccines. At the genomic level, these differences can be greater or equal to 5% in the hypervariable region of the S1 gene. These variant strains are usually restricted to geographic regions and most of the time are transient (Gallardo *et al* 2016), reason why diagnostics and epidemiological surveillance are crucial to determine their existence and persistence. This strategy helps planning preventative measures in the field.

IBV EVOLUTION

Viral evolution depends on two separated and independent mechanisms described by Mayr (Mayr 1988): (a) Generation of diversity, in which genetic/phenotypic variants are generated and serve as material for (b) selection, the virions generated after the replication process are released in the environment, and the survivors will serve as the genetic pool for subsequent generations (Gallardo *et al* 2010). IBV variability is generated by insertions, deletions and point mutations in addition to recombination events (Toro *et al* 2012). Jungherr and collaborators reported viral variability between IBV isolates for the first time in 1956. The described serological variations differentiated Massachusetts and Connecticut IBV serotypes (Jungherr *et al* 1956). IBV selection has been proved previously by different research groups (McKinley *et al* 2008, van Santen and Toro 2008). The rapid evolution ability is what makes IBV highly successful in the environment and is the reason why IBV continues to spread and circumvent vaccination programs used in the poultry industry (Toro *et al* 2012). Nowadays, we recognise the existence of dozens of IBV serotypes and even more genotypes and variants. Variants have been detected all over the world: Latin America, associated with kidney lesions (Hidalgo *et al* 1986); Africa, associated with swollen head syndrome (Morley and Thomson 1984); Egypt, associated with enteric disease (El-Houadfi *et al* 1986). In Asia, variants have been present since at least 1979 (Lohr 1988). In Australia, where evolution has been independent from other countries in the world (Ignjatovic *et al* 2006), variant IBV strains have been isolated since 1960 (Cumming 1963).

The introduction of exotic genotypes into a geographic region or country can increase the local genetic pool of IBV strains. This situation increases the chances of genetic recombination with local IBV strains and potentially generates IBV variants. The introduction of these IBV strains can be accidental, via contaminated poultry

products and lack of biosecurity, and/or premeditated. The most common premeditated introduction is caused when vaccines from exotic genotypes are introduced as a tool for controlling IBV outbreaks caused by variant strains. There are several examples of generation of variant IBV strains after the premeditated introduction of exotic genotypes. This review will focus on two cases. The first example is the event reported by Lee and Jackwood, where they describe the generation of the IBV genotype GA98 after the introduction and use of the DE072 vaccine. The IBV GA98 was the causal agent of extensive and costly outbreaks of IB in broilers in the state of Georgia (Lee and Jackwood 2001). The second example is the introduction of the IBV vaccine type 793B, 4/91 or 1/96, a UK-originated variant, to countries in Europe (Franzo *et al* 2014, Moreno *et al* 2017), Middle East (Tatar-Kis *et al* 2014) and Latin America (Sesti *et al* 2014, de Wit *et al* 2017). The reported consequences in Italy and Spain have been the recombination between local strains and the introduced vaccine (Moreno *et al* 2017).

VACCINES AND THEIR USE

Vaccines capable of inducing cross protection against different genotypes of IBV are of paramount economic and practical importance (Gelb 2018). Some commercially available vaccines are heterogenous, meaning that the predominant subpopulations in the vaccine are diverse and do not induce protective immune responses in chickens (Ghetas *et al* 2014). The best example of these heterogeneous vaccines is the ArkDPI vaccine. ArkDPI was originally isolated from the Delmarva peninsula and partially attenuated at the University of Delaware by 50 passages in embryonated eggs (Gelb and Cloud 1983). Then, the strain was distributed to different vaccine companies where they were further attenuated to produce different vaccines. The heterogeneity of the S gene in vaccines like ArkDPI might play the role of a virulent strain in terms of genetic variability, resulting in the emergence of new variants after replication, generation of diversity and selection. In addition, recombination of vaccine strains with local strains might also play a role. ArkDPI vaccines showing higher heterogeneity have been associated with respiratory signs and tracheal damage (Ndegwa *et al* 2012). Studies in variant IBV variability have shown different levels of heterogeneity in different IBV genotypes (Gallardo *et al* 2016). It is important to understand the heterogeneity of variant IBV strains before selecting them as vaccine candidates. Vaccine homology will also play a role. Heterologous vaccines will either provide no protection or, at best, partial protection. If partial protection is achieved, it will ameliorate clinical signs but will not reduce shedding of challenge/field virus. High loads of wild-type IBV strains combined with vaccine viruses creates the perfect scenario for variant virus generation. In summary, as it has been reported in the literature, live

attenuated vaccine usage has a major role in the genetic profile of IB strains isolated in the field (Jackwood and Lee 2017).

Other than vaccine selection, vaccine application is crucial to avoid variant IBV generation. Currently available live attenuated IBV vaccines are mostly applied in the hatchery at day of age and by spray or drinking water between 10 to 15 days of age in broilers and at least 3 to 4 times in layers before the laying onset. Massive application strategies for IBV vaccination are partially inefficient and usually result in vaccination failure (Jordan 2017). An example of partly ineffective vaccination methods are spray cabinets. Although the spray vaccination processing does not seem to damage the IBV virions, there is a significant titer reduction, most likely associated with the mechanical force applied to the virus particle during the vaccination process (Roh 2014). In addition, viruses are greatly wasted in the environment, especially when volumes as low as 7 ml per chick box are used (Jordan 2016). These issues are responsible for poor coverage during spray cabinet vaccination. Similar issues happen when using drinking water vaccination in the field, where poor vaccine coverage allows vaccine recirculation and rolling reactions. These rolling reactions allow the virus to mutate in every replication cycle while poor coverage allows the entrance of field viruses into the flock, creating recombination opportunities and subsequently generation of variant strains. All these problems are aggravated if half or quarter doses are applied.

POPULATION IMMUNE STATUS

Population immune status plays a crucial role in the evolution of IBV, not only because of the effect of vaccination in selective pressure but also because of immunodepression that will allow the virus to evolve freely in the affected population. Numerous epidemiological studies using conventional and molecular virology techniques have demonstrated the capabilities of IBV to circumvent vaccination programs which have been implemented since 1950 (Toro *et al* 2012). In particular, it is interesting to analyze Ark-serotype attenuated vaccines, where viral populations different from the vaccine emerge after vaccination causing disease and poor immune responses in vaccinated chicken flocks (McKinley *et al* 2008, Toro *et al* 2012). In the absence of immune pressure due to vaccination, IBV nucleotide changes in the S1 gene occur at a rate of 10^{-3} substitutions per site per year (Cavanagh *et al* 1998). GA98, which emerged due to the use of DE072 vaccine, showed mutation rates of 10^{-2} substitutions per site per year in the hypervariable region (HVR) of S1 (Lee *et al* 2001). Immunocompetent chickens show normal immune responses that are able to restrict viral replication and limit generation of genetic variants for selection. In chickens showing less than optimal immune responses, the vaccine viruses are able to replicate in a larger number

of individuals and viral populations different from the challenge strain may become predominant (Gallardo *et al* 2012, Toro *et al* 2012). This phenomenon can particularly happen when chickens are infected by highly prevalent and ubiquitous immunosuppressant viruses, i.e. chicken anemia (CAV) and infectious bursal disease virus (IBDV). Infectious bronchitis infection course in CAV or IBDV immunosuppressed flocks is longer, the clinical signs are more severe and the virus is able to persist for longer periods in the environment, facilitating diversity generation and subsequently emergent variant IBV strains (Gallardo *et al* 2012).

DETECTION, SURVEILLANCE, CONTROL AND PREVENTION

DETECTION AND SURVEILLANCE OF IBV

While we are very informed about the IBV strains affecting chickens in the U.S. and most of the countries in the E.U. due to continuous surveillance, there is lack of information in several countries in the world. In countries where surveillance is performed, most of the genotypes detected are not indigenous variants (de Wit *et al* 2010), but rather genotypes derived from the evolution of prevalent viruses and vaccines used in the region. Poor diagnostic and surveillance capabilities are a reality in most Latin American and African countries and, where these tools are available, it is very common to see errors in technique and result interpretation (unpublished data). Accurate diagnosis and targeted surveillance is crucial to adequately prevent and control IBV variant rise and its detrimental effects (Gallardo *et al* 2016). Other than orienting in the vaccine selection and vaccination strategy, accurate diagnosis will drive efforts to impede variant generation. IBV diagnostics are based on: (1) detection of the viral genome by molecular methods, such as reverse transcriptase polymerase chain reaction (RT-PCR) or RT quantitative PCR (RT-qPCR), targeting conserved genes such as N or M (Gallardo *et al* 2010, de Quadros 2012) and (2) conventional virology methods, such as virus isolation, serology and virus neutralization (Villegas and Alvarado 2008). A combination of these techniques is used in several laboratories around the world for diagnosing the disease. For surveillance, serum neutralisation in embryonated eggs, haemagglutination inhibition and sequencing of a portion or full length S gene amplified by RT-PCR are the most commonly used techniques (Villegas and Alvarado 2008, Gallardo *et al* 2010). Serum neutralization is used to type IBV using serotype-specific antibodies. However, hyperimmune sera are not readily available when new IBV variants arise. In addition, serum neutralisation test is expensive due to the use of a large number of SPF eggs. A similar problem is observed with HI, where specific reagents are needed. In addition, HI requires the treatment of the IBV isolate with neuraminidase since IBV does not have haemagglutination

capabilities naturally. Nowadays, the most commonly used technique is the RT-PCR to amplify the HVR of the S1 gene followed by sequencing of the amplicon, with or without prior virus isolation (Gallardo *et al* 2010). Virus isolation, if successful, increases the number of viral particles and consequently the amount of template for the polymerase reaction. The drawback of previous virus isolation is that *in vitro* replication allows viral mutations in the IBV genome, increasing variability during the virus adaptation to the embryonated egg. RT-PCR directly from chicken specimens followed by sequencing, even though less sensitive, is the best method to perform surveillance. This methodology will provide genetic information about the wild-type virus without the variability added during virus isolation. The nucleotide heterogeneity of S1 is largely contained within three different HVR's. Those HVR's are located in amino acids 38 to 67, 91 to 141 and 274 to 387 (Cavanagh *et al* 1988). Complete or partial analysis of the S1 has been used to determine IBV genotypes. Currently, nearly 100 genotypes have been recognised for causing impact on the commercial poultry industry. Some are restricted to specific geographic areas (Gallardo *et al* 2016).

The size of the S1 fragment amplified by RT-PCR for surveillance is of major importance. While using the full S1 is the best choice because it allows the analysis of all three HVR's, this technique is less sensitive than amplifying a shorter fragment of S1 and requires a considerable amount of well-preserved viral RNA in the sample. On the other hand, while amplification of small segments (between 300 and 500 bp) increases the test sensitivity, short sequences overlook variations in other portions of the HVR's or recombination sites (Moreno *et al* 2017). While not as accurate as using the full S1 gene, the amplification of a segment of 750 nucleotides has been proven to be adequate for IBV surveillance and evolution studies *et al* 2012). Inconsistencies in phylogenetic analyses makes IBV genotyping and characterisation difficult. Valastro proposed a classification system using full S1 sequences (Valastro *et al* 2016). The reasoning behind this classification is that there is lack of consistency when using only one or two of the three HVR's of the S1 gene in the analysis. In addition, the spatial component of the characterisation shows evidence that most of the current strains originated from old lineage IBV's and their rapid evolution assures the discovery of new genetic variants in the future. Full genome characterisations are useful when available. The information provided by whole genome sequencing should confirm the partial or complete analysis of the S gene and provide further information on other genes. Variability of the outer viral proteins such as the spike and envelope are of particular interest.

SURVEILLANCE INTERPRETATION

The routinely performed genotypic surveillance of IBV involves molecular amplification and sequencing of

a portion of S1 or the complete S1 gene. These sequences are curated and compared with sequences located in databases such as GenBank. The sequences should be compared in phylogenetic trees with the most commonly used vaccines and strains isolated in the geographic area of interest. The size of the segments compared are of major importance. Short sequences do not account for all HVR's and might provide inaccurately high homologies when compared to other sequences in the database, leading to wrong interpretations. In addition, comparing segments of different lengths can generate high identities with low query coverages, resulting in biased homologies that need to be considered in the interpretation. If the coverage is low, the confidence of the analysis might be at risk. The detection of a genetic variant does not imply that this virus is the causal agent of problems in the field. Surveillance data should be correlated with clinical signs, pathology, management, field data and vaccination records. Not all variants acting in a geographic region require the preparation of autogenous vaccines since a percentage of them are transient and restricted to a limited geographic area. It is important, other than performing molecular epidemiology using phylogenetic trees, to associate these trees with pathological observations in the field, perform virus isolation and biological characterisation in chickens. If the viruses are highly pathogenic or cause severe productive losses, vaccine protection studies are recommended. The objective is to test protection elicited by commercially available vaccines. If the protection provided by the available vaccines is not adequate, studies to assess variability of the detected variant should be performed to determine if formulating a homologous vaccine to prevent outbreaks is safe (Gallardo *et al* 2016). Sometimes IBV variants that are not clearly associated with disease are detected. In this case, it is recommended to continue with close epidemiological surveillance. Generation of databases for specific regions is desired, since they allow for better interpretation of results (Gallardo *et al* 2016).

CONTROL OF IMMUNOSUPPRESSIVE VIRAL DISEASES

Immunosuppression in poultry can be caused by several factors, including stress, nutritional deficiencies, mycotoxins and viral diseases (Hoerr 2010). The main viral immunosuppressive diseases reportedly linked with IBV cases in the field are IBDV and CAV. IBV cases in broiler chickens usually occur between 35 and 49 days of age (figure 1) and are potentially associated with immune deficiencies (Toro *et al* 2006, Gallardo *et al* 2016). As a confirmatory experiment, bursa of Fabricius samples were collected from a representative number of flocks in a broiler integrator in the U.S. Each bursa was divided in half, one half was used for IBDV viral load measurement by RT-qPCR and the other half was used for bursal histomorphometry to evaluate lymphoid depletion (Gallardo 2018) (figure 2).

Results showed a peak of IBD viral load between 25 to 30 days of age, followed by a peak of lymphoid depletion in the bursa around day 35 of age. These results correlate with the reported age of highest incidence of IBV cases in broiler chickens (figure 1) (Gallardo *et al* 2016). This correlation was previously reported by Toro *et al* (2006) where they measured lymphoid depletion scores of bursa and thymus sections in 322 cases of diagnosed IB between 1997 and 2002 in Alabama. In that project, peaks of lymphoid depletion associated with IBDV and CAV in bursa and thymus, respectively, were found in broilers between 30 and 40 days of age (Toro *et al* 2006). IBDV and CAV immunosuppression can be prevented by vaccination

strategies. An efficient vaccination strategy for IBDV prevention must involve hyperimmunisation of breeders using live attenuated or recombinant and inactivated vaccines in order to protect chickens until approximately 3 to 4 weeks of age. In addition, vaccination of the progeny with recombinant, live attenuated or antigen-antibody complex IBDV vaccines provide appropriate protection until approximately 5 to 6 weeks of age in broilers (Gallardo 2018). For CAV, an effective preventative strategy is to generate adequate immune response in the breeders to impede vertical transmission and to transfer maternal antibodies to the chicks. For that, an adequate vaccine should be selected and the induced immunity should be

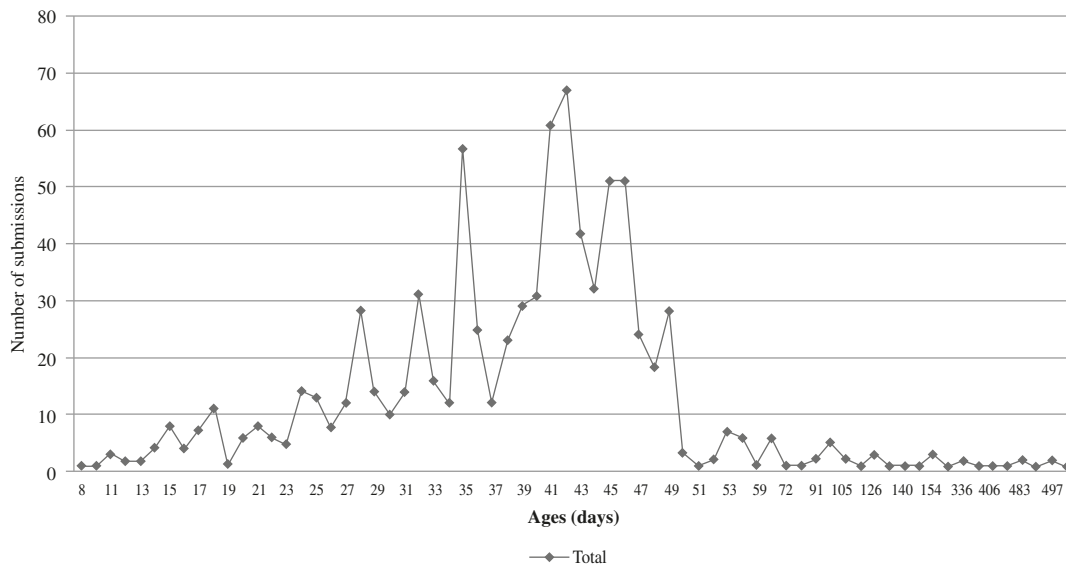


Figure 1. Age of occurrence of IBV respiratory cases in broilers and layers (n:1444) in California between 1997 and 2012.

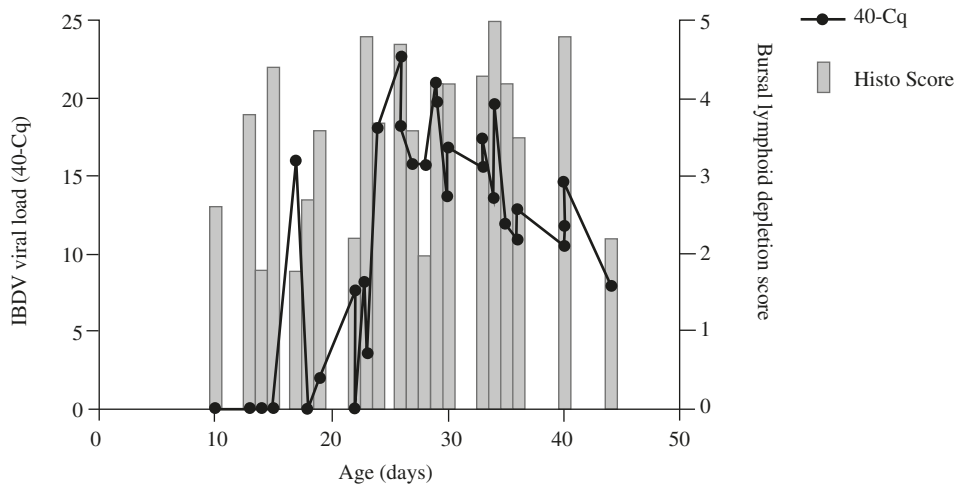


Figure 2. Bursa of Fabricius lymphocyte depletion scores from 0 to 5. (0: no lymphoid depletion to 5: highest level of lymphoid depletion) and viral load in bursas expressed as 40-Cq. These data were obtained from commercial broiler chickens at different days of age starting at 10 days and ending at 43 days.

periodically assessed (Gallardo 2018). There are commercial ELISA kits available for CAV antibody assessments. Serum samples diluted 1:100 allow clear dispersion of the data points (Toro 2015). The results should be analysed considering S/N ratio limit values of 0.2 and 0.8. Values below 0.2 represent protective titers, values between 0.2 and 0.8 represent low titers and values above 0.8 represent non-protected chickens or negative titers (Toro 2015). Even though it has not been reported as a direct cause of increased IBV cases, Marek's disease virus (MDV) can cause immunosuppression, affecting both humoral and cellular immune responses (Gimeno and Schat 2018). The MDV immunosuppression pathogenesis is very complex, poorly understood and in many cases under diagnosed. MDV vaccination protects against some aspects of viral immunosuppression but certainly not all (Gimeno and Schat 2018).

IBV MANAGEMENT

Proper management is a key component of a disease-free flock. Chickens can be exposed to stressors and infectious diseases that impair innate and acquired immunity, eroding general health and welfare (Hoerr 2010). Any stress caused by poor management can affect the immune system and the consequences can be linked to upper respiratory tract infections (Hoerr 2010). An example of poor management is poor ventilation. If intensive productive units are poorly ventilated, the incidence of respiratory diseases increases. Increased particulate matter and ammonia in the air (Shepherd *et al* 2015, Zhao *et al* 2015), in addition to the increased concentration of Gram-negative bacteria in the environment (Zhao *et al* 2016), can cause irritation, inflammation and changes in the microbiota, inducing complex upper respiratory diseases (figure 3) (Gallardo 2018). Addressing the presence of risk factors and understanding their interaction is essential for a successful management and consequently optimal health and welfare. In addition, genetics and nutrition have a major role in an efficient production system (Hoerr 2010). Food-borne mycotoxins and suboptimal nutrition can diminish immune responses, particularly the innate immunity to pathogens (Hoerr 2010). Biosecurity is the first barrier for pathogen introduction in poultry flocks. Lack of or flawed biosecurity facilitate the introduction of IBV, immunosuppressive pathogens (CAV, IBDV, MDV, reoviruses and adenoviruses) and other respiratory viruses such as avian influenza, Newcastle disease and avian metapneumoviruses. These upper respiratory tract viruses affect the ciliated epithelium and mucous glands of sinuses and tracheas, predisposing the entrance of more pathogens.

In conclusion, IBV is highly variable and variants arise because of the constant evolution of this virus. There are factors that predispose the virus to more variability, such as the introduction of exotic genotypes, poor vaccine selection and application and immunosuppressive diseases.

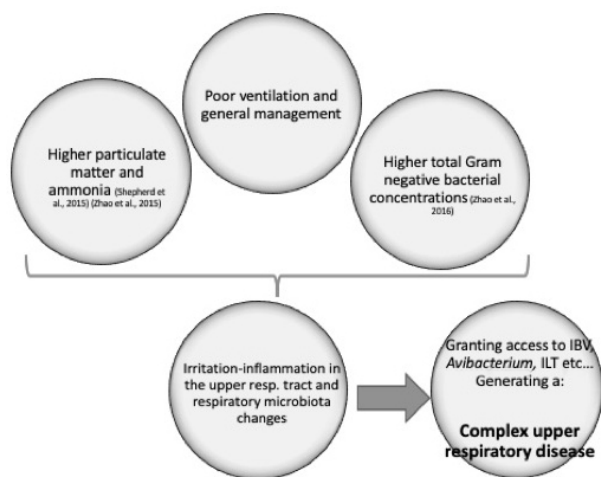


Figure 3. Effects of poor management in the air quality and upper respiratory tract of chickens, pathogenesis of complex upper respiratory disease.

Diagnosis, detection and surveillance are necessary to investigate and properly analyse the circulating IBV genotypes. This understanding depends on appropriate interpretation of results and assists the development of suitable preventative strategies and vaccination decisions. In addition, good practices, management and adequate control of immunosuppressive diseases help preventing the arise of variant IBV strains. Prevention of endemic diseases in poultry is based on properly using the available diagnostic and surveillance tools and interpreting the results obtained. When these tools are not properly used, misleading information is shared, and deceptive epidemiological data is reported. These events can affect future preventative strategies and predictions on IBV evolution.

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