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Biotic concerns in generating molecular diagnosis matrixes for 4 avian viruses with emphasis on Marek's disease virus

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ABSTRACT

The great advance in the field of diagnosis of avian viruses is reflecting the highly sophisticated molecular assays of the human and general virology in providing highly sensitive and fast methods of diagnosis. The present review will discuss the biotic factors and the complexities that became evident with the evolution of the novel molecular diagnostic assays with emphasis on 4 avian viruses, chicken anemia, infectious laryngotracheitis, turkey meningoencephalitis, but mainly on Marek's disease virus.

To create a biologically meaningful diagnosis, attention should be dedicated to various biotic factors and not only of the diagnostic assay. Included among the important factors are, (a) the sample examined and the sampling strategy, (b) the outcomes of the pathogen amplification ex vivo, (c) the sampling time and its reflection on the disease diagnosis, (d) the impact of simultaneous multiple virus-infections regarding the ability to demonstrate all pathogens and inter- and intra-interactions between the pathogens. A concerted consideration of the relevant factors and the use of advanced molecular diagnostic assay would yield biologically significant diagnosis in real-time that would beneficiate the poultry industry.

1. Introduction

An enormous progress occurred in the field of diagnosis of avian viruses during the last two decades. The transition from classical methods to the era of molecular biology was exponential in the creativity of new assays that were based on revolutionary technologies. The novel molecular assays offered numerous advantages as compared to the former approaches, mainly in shortening the time required to obtain the results, but also in the sensitivity of virus detection. However, with the switch to fast and sensitive diagnostic assays various factors have to be taken into account to obtain a biologically significant diagnosis. The present review will expose several aspects that deserve specific consideration in the diagnosis of avian viruses by molecular assays.

Beginning from the year 1970, with the discovery of cell culture, many avian viruses were grown *in vitro*. In comparison with the use of eggs and animals, virus growth in cell culture became more convenient and cost effective. This method is considered gold standard for virus isolation and identification. Classical approaches for the detection and diagnosis of avian viruses were based on their replication in embryonated eggs, their cultivation in cell-cultures, and their ability to haemagglutinate red blood cells. Afterwards, the avian virus specific detection was facilitated by morphological alterations of the developing embryo, by haemagglutination inhibition of virus by specific antibodies, antibody detection in sera by ELISA, immunofluorescence and by other serological assays (Williams, 2016). These assays were based on the virus ability to replicate in experimental systems. The secondary amplification system might differ from the virus natural milieu, the chicken body, and might lead to artifactual outcomes, as will be discussed in the present review. The development of the revolutionary concept of demonstrating the pathogen nuclei acid by various assays brought a new era in the development of novel diagnostic assay, however with certain advantages and disadvantages. The latest molecular diagnostic technologies are more advantageous as they offer more sensitive, less time consuming and high throughput results. The novel technologies include the polymerase chain reaction (PCR), Reverse Transcriptase PCR (RT–PCR), real-time PCR (qPCR), DNA probe, nucleic acid sequencing *etc.*. Nowadays, no diagnostic area cannot be imagined without molecular amplification assays. As a result, at the time of writing this review article, a PubMed search of "diagnosis of avian viruses by PCR" resulted in 721,000 hits, including all avian viruses and various combinations of assays. As it is impossible to cover the field, so in that review will mainly describe the process of incorporating the molecular amplification for the detection of avian oncogenic viruses.

Recently Shojaei et al. (2015) and Astill et al. (2018) described novel methodologies developed to detect rapidly avian viruses, such as

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biosensors, wearable poultry sensors, and noninvasive non-contact analysis mechanisms, such as a vocalization analysis and various imaging techniques. The specificity and speed that the biosensor diagnostic technologies presented are promising. However, the availability of these sophisticated means of diagnosis requires professional input and consideration in order to obtain meaningful findings. In addition to providing data to producers on animal health status in real-time, these detection methods are beneficial as they decrease the need for humans employed in monitoring of poultry houses, thereby decreasing the chance for introduction of infectious agents into poultry flocks and exposure to zoonotic pathogens.

The present review will highlight several biotic factors and complexities, as well as the consequent approaches that were taken regarding avian viruses, in view with the evolution of the diagnostic assays. Concomitantly with the development of the new methods, the concept of diagnostic sampling evolved based on experiences gained from the diagnosis of commercial poultry by molecular assays. In addition, complexities that might be considered with the transition of poultry virus diagnosis towards advanced assays will be critically reviewed. The concepts conferred in the present review will be illustrated mainly by the studies on three avian vaccine viruses, Marek's disease (MDV) (Schat and Nair, 2013), (Family: *Herpesviridae*, Subfamily: *Alphaherpesviridae*, Genus: *Mardivirus*, Species: *gallid herpesvirus 2*, GaHV-2, ICVT, 2017) chicken anemia (CAV) (family *Circoviridae*, genus *Gyrovirus*) (Schat and Van Santen, 2013), Infectious Laryngotracheitis (ILTV) (Garcia et al., 2013), (Family: *Herpesviridae*, Subfamily: *Alphaherpesviridae*, Genus*:Iltovirus*, Species: *Infectious Laryngotracheitis* virus) and the turkey flavivirus, Turkey Meningoencephalitis Virus (TMEV) (Iancunescu, 1976) (genus *Flavivirus* of the family *Flaviviridae*). However, diagnostic complexities of additional avian viruses will be discussed, such the poultry respiratory viruses, Avian Influenza (AIV) (Swayne et al., 2013), (Infectious Bronchitis Newcastle disease (NDV) (Miller and Koch, 2013) (Family: *Paramyxoviridae*, Subfamily: Avula*viridae*, Genus*: Newcastle disease virus*), Infectious Bronchitis (IBV) (Jackwood and de Wit, 2013) (Family: *Coronaviridae*, Subfamily: Corona*virinae*, Genus:Gammacoronvirus, Species: *Avian coronavirus*), and others.

Marek's disease virus (MDV) (Schat and Nair, 2013) include 3 distinctive serotypes, affecting chickens and turkeys. Most MDV serotype 1 viruses are virulent, as they infect and transform T-lymphocytes. MDV-1 induced clinical and sub-clinical infections such as neurological symptoms, tumors and immunosuppression. The virus replicates in the feather follicle epithelium and spreads horizontally in poultry house with dust and dander. Major morbidity and mortality associated with MDV-1 led to intensive effort during the 70′ to develop vaccines. Since then the disease is controlled worldwide by vaccination of day-old chicks or 17–18 embryonic day embryos *in ovo* with live avirulent and nononcogenic vaccine strains of the three serotypes. The serotype 1 MDV vaccine, denoted CVI988/Rispens (de Boer et al., 1986; Rispens et al., 1972), developed from a naturally-attenuated MDV-1 strain, is currently the most effective and widely used vaccine. The serotype 2 MDV vaccine (SB-1) (Schat and Calnek, 1978), also derived from a chicken naturally avirulent isolate and serotype 3 vaccine, developed from a herpesvirus of turkeys (HVT) (Okasaki et al., 1970) are also in use.

CAV infections have serious economic impact on the poultry industry. The infection is manifested with evident disease or as a subclinical infection and immunosuppression, reflecting negatively on diseases caused by other pathogens, and affecting vaccination efficacies (Schat and van Santen, 2013). In young chicks, the infection may display with various signs with various degrees of severity. These signs include stunting, runting, increased mortality, anemia, bone marrow cell depletion, subcutaneous hemorrhages and atrophy of secondary lymphatic organs such as the thymus, bursa and spleen. These effects are caused because of the multi-potent efficacy of CAV to infect stem cells of both the hematopoietic and lymphocytes cell lineages in the bone marrow and thymus. Clinical signs do not appear always, and sometime are barely evident, because of age-associated resistance and/or immune system maturation. Infection of commercial chicken flocks in Israel with CAV showed that CAV infection correlates with a decline in productivity (Davidson et al., 2004a,b).

The herpesvirus ILTV causes severe respiratory disorder and latent infections with concurrent reactivations due to stressful physiological conditions through lifetime (Garcia et al., 2013). ILTV live vaccines are administered *via* eye-drop, spray or drinking water. Alternatively, the cloacal vent-application has been successfully practiced in Israel (Samberg et al., 1971) until recently when ILTV vaccine administration was changed to the drinking water route. Infectious laryngotracheitis (ILT) is a respiratory disease of poultry caused by an alphaherpesvirus, ILTV. The disease severity varied from mild to acute with mortality rates that can vary and reach up to 70%, depending on the virulence of the infecting virus. The milder form of ILT is manifested with nasal discharges, conjunctivitis, and reduced egg production, whereas in severe forms of ILT the clinical signs include gasping with efforts to inhale, coughing, excretion of bloody mucus, dyspnea up to suffocation and fast mortality.

The turkey flavivirus TMEV causes an economically-important neuroparalytic disease leading to paresis, in-coordination and drooping wings. The TMEV-induced disease is controlled by vaccination with a live attenuated virus (Iancunescu et al., 1975).

2. The diagnostic samples

2.1. Tissue selection

In contrast to the diagnosis of avian viruses by classical assays that employed blood, plasma and tissue homogenates, the molecular diagnosis employed directly the target organ for the virus replication *in vivo*, as a source for nucleic acid to demonstrate the virus presence.

In the case of the avian oncogenic viruses, the best sample are the tumor-affected organs, or the lymphatic organs, which contain the highest numbers of virus-transformed lymphocytes. With that concept, the diagnostic PCR amplification of MDV, and the retroviruses reticuloendotheliosis (REV) (Family: *Retroviridae*, Subfamily: *Orthoretrovirinae*, Genus, *Gammaretrovirus*, Species: *Reticulendotheliosis virus*) and avian leucosis (ALV) (Family: *Retroviridae*, Subfamily: *Orthoretrovirinae*, Genus, *Alpharetrovirus*, Species: *Avian Leukosis virus*) was performed on DNA purified from spleen and liver of diseases chickens (Davidson et al., 1995a,b). However, for the diagnosis of the specific neurological manifestation in chicken infected with virulent MDV in the early stages on infection, the transient paralysis, we envisaged that the affected chicken brain would contain a higher number of virions than the blood and spleen, therefore, the brain was demonstrated as the preferred site for MDV-DNA (Davidson et al., 1998). A similar approach was engaged for the direct demonstration of the turkey flavivirus in the brain tissue of paralysed turkeys (Davidson et al., 2000). In the case of ILTV infection, where the ILTV target cells for replication were the epithelium lining inside the trachea, often causing exudates, the trachea content was initially used as the source for DNA purification to amplify the ILTV from clinically-affected commercial chickens (Davidson et al., 2009). avian

Another example would be the complex of avian respiratory viruses, AIV, NDV, avian pneumovirus (APMV) (Family: *Paramyxoviridae*, Subfamily: *Pneumovirinae*, Genus: *Metapneumovirus*, Species: *Avian metapneumovirus*) and the Turkey Rhinotracheitis virus (TRTV) (Swayne et al., 2013; Miller and Koch, 2013) (Family: *Paramyxoviridae*, Subfamily: *Pneumovirinae*, Genus: *Metapneumovirus*, Species: *Turkey Rhinotracheitis virus*). To demonstrate the presence of these viruses, direct sampling from trachea and cloaca of suspected birds is performed using dedicated swabs (Williams, 2016). However, for the collection of virus isolates, their use in experimental infection trials and sequencing, the respiratory viruses are further cultivated in embryonated eggs or in

tissue cultures. As RNA viruses are mutating at a high rate upon each replication cycle, drifts, namely 10^{-4}-10^{-6} mutations/ nucleotide/replication cycle, (Duffy et al., 2008), ex vivo amplification causes *de novo* genomic changes that were not reflect on the original isolate. Moreover, in the case of AIV, genomic exchanges can occur between fragments, shifts, leading often to the creation of new AIV strains (Swayne et al., 2013).

We envisaged the possibility of using feathers for direct diagnosis and research because their great advantages; sampling of feathers is non-invasive, it is a relatively easy and convenient procedure, as bleeding and necropsy could be avoided. In MDV research the feathers were emphasized, since this is the only site where fully infectious viruses are produced in the chicken. In fact, MDV was the first avian virus described in the context of its presence in feathers and was extensively studied (Schat and Nair, 2013). As MDV became the prototype avian virus which reside in the feather follicle epithelium and shafts, the feather tips were incorporated for use in the diagnosis of commercial flocks (Davidson and Borenshtain, 2002, 2003, Davidson and Shkoda, 2005; Davidson, 2009a). Ralapanawe et al. (2016) studied commercial layers demonstrating the MDV-1 in feathers and dust. However, monitoring the virus presence in feathers illustrate more accurately the current infection status than dust, because dust reflects cumulatively all the birds in the poultry house over a prolonged time-period. CAV detection in the feather tips was firstly reported in experimental infection trials (Davidson et al., 2008a,b), and then applied for diagnosis (Davidson et al., 2013). The ILTV viremia could also be demonstrated in the feather shafts of commercial ILTV-infected chickens (Davidson et al., 2009c; Davidson et al., 2016). TMEV was the first turkey virus that could be detected in the turkey feather shafts (Davidson et al., 2017b)

The correlation between sampling feathers or pooled visceral organs for the detection of MDV and for CAV in commercial flocks was analysed. We included samples submitted for diagnosis from various poultry flocks and of various lines and ages. The DNA samples from pooled organs and feathers of the same birds were amplified by real-time PCR and plotted to verify the linear correlation between the amplification values obtained with the two samples. The samples examined for MDV presence originated from 86 flocks in which the amplification values obtained with organ and feather tip DNAs correlated linearly at a high level of statistical significance ($p < 0.001$). The samples examined for CAV presence originated from 95 flocks in which the amplification values obtained with organ and feather tip DNAs also correlated linearly at a high level of statistical significance ($p < 0.001$) (Davidson I., unpublished).

The viremia stage of infection with additional viruses that spread systemically could be traced by analysing feathers; the replication of the highly pathogenic avian influenza was demonstrated in the feather epithelium cells of call ducks through the natural infection route, and these feathers could be used to create a re-infection of ducks, suggesting that feathers could be a potential source of infection for unaffected birds in nature (Yamamoto et al., 2007). Further, replication and ultrastructural changes of two strains of H5N1 AIV were verified in domestic ducks and geese (Yamamoto et al., 2008).

The viscerotropic velogenic NDV, genotype VII was also detected by RT qPCR and immunohistochemistry, suggesting that feathers act as sources of viral transmission. (Lee et al., 2016).

2.2. The sample size

The diagnosis accuracy of avian viruses depends on the sample size, which in turn, determines the sensitivity and specificity of the virus detection. A small sample size produces an imprecise estimate of accuracy with wide confidence interval which is non-informative for decision makers, while large sample size requires extensive resources. Unfortunately, analyzing sample sizes did not attracted much attention, especially when molecular diagnosis was employed. A special attention to the sample size has been focused in studies on human viruses, where the number of person included has a great impact of the findings significance (Hajian-Tilaki, 2014). Formula for sample size calculation in case of individual humans or animals with regards to sensitivity and specificity analysis. The tables were derived from formulation of sensitivity and specificity test using Power Analysis and Sample Size (PASS) software based on desired type I error, power and effect size (Bujang and Adnan, 2016).

Most of the current diagnosis of avian viruses is performed by molecular assays which require very small amounts of nucleic acids. As the required amounts can be purified from a small quantity of tissue, a special attention has to be devoted to accurate representation of the tissue. A common belief and practice is to prepare a small sample for molecular analysis, even few cells, as only a minute nucleic acid quantity is needed in molecular biology. However, to obtain a wide dynamic range of the molecular diagnosis, it is recommended, therefore, to purify nucleic acids for molecular assays from a bigger sample, and to use as much as needed for the use in the molecular assay.

For that reason the DNA purification for the diagnosis of avian oncogenic viruses was performed from a pool of several sections of frozen tissues of 3–4 birds (Davidson et al., 1995a,b). Since the diagnosis of commercial flocks is based on the amplification of both pooled visceral organs and feathers, the tips of 2–3 feathers from each of 3–4 bird serve to create a pool for DNA purification. Moreover, when feathers were sampled for determination of the vaccine application efficacy, between 3–5 different birds were sampled and pooled each time in order to create a wider diagnosis (Davidson et al., 2018a,b).

2.3. The sampling timing

The timing of sampling is important for the demonstration of the virus presence. The experimental infection trials that are performed according to the experimentally-established infection model provided information about the kinetics of the poultry virus presence, in the target organ of replication, or in the bloodstream, as viremia. For example, the respiratory viruses AIV and NDV can be detected for about 5 and 10 days post-infection (Davidson et al., 2017a). Looking for virus demonstration at a later time might be unsuccessful, depending on the virus biology, virulence and kinetics of replication.

The particular infection form of latency or pseudo-latency might also lead to difficulties to detect the infecting viruses. In these circumstances the infecting viruses are present in distinctively minute amounts, at least until reactivation, as in the case of herpesviruses, like ILTV (Guy et al., 1991; Hughes et al., 1991) and circoviruses, like CAV (Miller et al., 2008). In commercial flocks the infection is not synchronous, and the timing of sampling is dictated by the appearance of clinical signs in conjunction to other factors. Inability to demonstrate the infecting virus that mainly reflects the acute phase of infection and is characterized by systemic viremia, unlike in the case of sub-clinical infections, where the virus resides mostly latent in the target organs of replication.

2.4. The nucleic acid purification

An essential attention should be dedicated to the methods of nucleic acid purification directly from the origin bird organs, without further virus amplification in *ex vivo* systems. In spite of the enzymatic amplification advantages, the PCR assays are highly affected by inhibitory compounds that might hinder the real outcomes. Several compounds are recognized to inhibit the PCR, such as: hemoglobin, urea, heparin, organic or phenolic compounds, glycogen, fats, Ca^{2+} ions, laboratory gloves powder, and also various effects characteristic of various tissues, such as proteolytic enzymes that are abundant in faeces. To avoid the inclusion of such compounds in the analytical samples the nucleic acid purification has to avoid their inclusion. While in former years the phenol/chloroform nucleic acid purification methods were manually

performed, in the last years commercial purification kits are available. However, the users have to be extremely careful to select the most fitted purification kit for their needs.

For example, the avian system offers a unique example for the complexity of DNA purification from various clinical samples, and the inapplicable automatic use of the same purification methods. The poultry virology provides two distinctive examples for particular attention: (a) the DNA purification from avian red blood cells that contain nuclei, is a challenge as compared to the red blood cells of other species, that do not contain nuclei. The resulting purified DNA from avian red blood cells is viscous and requires enzymatic treatment with Proteinase K. (b) The DNA purification from solid tissues, like the visceral organs, skin lesions and feather tips required the optimization of the nucleic acid purification method, in term of suitability and sensitivity. In our studies the automated Maxwell® platform was used, where the feather tips, that are solid tissues, were comparable to mouse tails (Davidson et al., 2018). That system is advantageous for nucleic acid purification from solid tissues because it avoid the need to pulverize or to homogenize the analysed tissue, were only a small tissue piece is applied directly into the kit.

Additionally, a special care should be taken with the RNA purification due to its sensitivity to degradation. Small amounts of ribonucleases (RNases) can sometimes co-purify with isolated RNA and compromise downstream applications. Such contamination can also be introduced *via* tips, tubes, and other reagents used in the procedure.

3. Virus demonstration by molecular amplification

3.1. Conventional PCR

The history of the PCR assay has been described as a classic EUREKA moment, or as an example of cooperative teamwork between disparate researchers. The PCR technique, invented in 1985 by Kary B. Mullis, allowed scientists to make millions of copies of a scarce sample of DNA (Bartlett and Stirling (2003). Since the discovery of the PCR and RT-PCR the area of diagnosis incorporated the assays for the detection of the vast majority of pathogens, including the avian viruses.

In an attempt to provide a rapid and sensitive means of differential diagnosis we have applied the PCR for MDV and REV detection (Davidson et al., 1995a), and subsequently added ALV, LPDV and ALV-J env PCRs to the differential diagnosis scheme (Davidson et al., 1995b). In the early years we investigated the validity of PCR as compared to the classical diagnostic methods. On every avian disease diagnosed by PCR, we performed two or three analyses in parallel: virus isolation in tissue cultures and identification of virus infection by immunofluorescence staining of infected cells with specific monoclonal antibodies, and histopathology of the tumor. A complete resemblance was found among the various assays. However, PCR appeared to be the method of choice for diagnosing avian oncogenic viruses as it overcame the difficulties of differential diagnosis, and enabled the detection of multiple viral infections (Davidson et al., 1995a,b).

3.2. Real-time PCR

Following the revolutionary invention of the PCR by Kary Mullis in 1984 (Bartlett and Stirling, 2003) the real-time PCR (qPCR) has been developed, becoming a common tool for detection and quantification of genes and pathogens (Deepak et al., 2017). Unlike the conventional end-point PCR (cPCR), whose final amplicon generation was detected at the end of the enzymatic reaction, therefore denoted as cPCR, by the qPCR the products are evident during the reaction. The qPCR technology proved to be more sensitive, allowing the detection of only few target molecules even in a mixture of excessive high levels of background DNA. Moreover, the qPCR allows quantitative genotyping and detection of single nucleotide polymorphisms and allelic discrimination as well as genetic variations when only a small proportion of the sample carries the mutation. However, the development of qPCR is complex as, (a) it prerequisite accurate calibration for optimal performance, (b) use of adequate reference materials, (c) sensitivity of inhibitory compounds in the nucleic acid target sample, and more (Kralik and Ricchi, 2017). Since more than a decade ago Deepak et al. (2017) described the dramatic leap in the use and application of the qPCR in various areas of biology and infectiology in humans, plants and animals. Similarly to the cPCR, the avian virology incorporated the various qPCR technologies in the study of all the known avian viruses and bacteria, and several examples will be mentioned below.

A key biotic factor, that has to be evaluated when performing virus identification and quantification, which is whether the viral infection is acute or sub-clinical. The particular case of poultry infection with two ubiquitous viruses, MDV and CAV, which can produce both types of infection will be presented and can exemplify such complex situations.

Most of the diagnostic assays for the two viruses were calibrated using experimental infections or samples from clinical cases in commercial flocks (Davidson et al., 1995a,b, 2004a,b,2007). Until our study (Davidson et al., 2013) not much attention has been paid to detect subclinical infections, in particular, and to the quantitative detection of the two viruses in the organs of commercial chickens with or without clinical signs. Several reports described qqPCR to estimate MDV (Baigent et al., 2011; Cortes et al., 2011; Islam et al., 2004; Renz et al., 2006) or CAV (Markkowski-Grimsud et al., 2002) viral copies in the organs of infected birds or dust.

In our study (Davidson et al., 2013) the presence of MDV and CAV in feather tips and in pooled visceral organs, spleen and liver, by cPCR were comparable with the viral copy numbers as determined by qqPCR. Negative samples by cPCR contained low viral copy numbers by qqPCR, and positive cPCR samples had copy numbers $\geq$ 10,000. The correlation between clinical signs and the number of CAV and MDV genomes was evaluated. The genome numbers of MDV and/or CAV was variable among the different chicks, however, the average genome numbers in chicks with or without signs differed; in chicks with clinical signs about 10^2-10^3 more MDV and 10^5 more CAV viruses were detected. These findings confirmed previous studies demonstrating that appearance of visible clinical signs is correlative to the presence of numerous viral genomes (Islam et al., 2004, 2006). Accordingly, in sub-clinically infected chickens lower viral numbers were be detected. The disperse range of viral genomes in various commercial birds indicated that the viruses can be present in various loads, and that finding consisted a reflection of their ubiquitous nature.

The ability to quantitate the infecting viruses by their amplification, and the biological feature of the two avian ubiquitous viruses, MDV and CAV, that produce both acute and non-symptomatic infections, raised a biotic dilemma, “to quantify or not to quantify”. The problem regarding the biological significance of quantification embraces a broader context. The main factor is the nature of the infecting pathogen. In case that the infection was caused by a zoonotic pathogen that is not allowed to be present, like in case of infections with highly pathogenic avian influenza, or salmonella, the quantification is warranted to determine the status and level of infection, and to determine the freedom of these pathogens and efficacy of vaccination or of various disinfection means. When the infection involve ubiquitous viruses, performing quantification of infection is less critical, although it can provide information on the level of infectivity, as the pathogen copy number influences the severity of clinical signs. Generally, pathogen quantification can indicate the vaccination efficacy on the background of infection with virulent wild-type pathogens. However, the need for quantification performance is not trivial in cases of sub-clinical infections. The performance of pathogen quantification has to be associated with the establishment of regulations regarding the permitted levels of pathogen presence, that will be associated by decisions regarding the need to take actions. In these cases clear criteria and action policies have to be formulated.

3.3. Droplet digital PCR

The Droplet Digital PCR technology was developed to quantitate the target molecules in the diagnostic samples (Baker, 2012). The technique is based on the water-emulsion technology by which the DNA sample is fractionated in about 20,000 droplets. In each droplet an end-point PCR is conducted, afterwards the positive droplets are counted to reflect the absolute target quantification per input sample, without the need for standard curves, as in qqPCR. The high precision and sensitivity of the assay is higher than in qqPCR, and the assay performance is easier, as there is no need for multiple dilution steps. However, this assay was reported only for the detection of salmonella and campylobacter (Steele et al., 2018), for the detection of AIV infections with the highly pathogenic H5N1 (Hu et al., 2017) and the H7N9 subgroup (Yan et al., 2016) in humans.

3.4. The loop-mediated isothermal amplification

The Loop-Mediated Isothermal Amplification (LAMP) method is a fast (the assay often takes less than an hour), low cost, highly sensitive, highly specific and less laborious alternative to detect infectious disease agents. The LAMP assay is performed at a single temperature, around 60 °C, in a water bath or heating block. The sensitivity of this LAMP assay is very similar to end-point PCR with a similar detection limit. The assay preparation employs lyophilized reagents and visualization of results using hydroxynaphthol blue (HNB) or a UV lamp with fluorescent intercalating dye in the reaction mixture. LAMP is also less affected by the inhibitors present in DNA samples. The procedure can be completed under isothermal conditions so thermocyclers are not needed. The ease of use of the LAMP assay allows adaptability to field conditions and works well in developing countries with resource-limited laboratories. The assay is particularly robust because the reaction mixture preparation does not involve complex steps.

Many LAMP assays have been developed and validated for important epizootic diseases, including 18 viruses thought notifiable of ruminants, swine and poultry by the World Organization for Animal Health (OIE) (Mansour et al., 2015). The LAMP assay was also developed for detection of the Tembusu virus (Tang et al., 2016), four immunosuppressive viruses (Song et al., 2018), avian leucosis virus (Peng et al., 2015), avian reovirus (Kumar et al., 2017), and found to be useful also in the detection of MDV in feathers and internal organs of infected chickens (Woźniakowski and Samorek-Salamonowicz, 2014). Direct detection of MDV DNA in poultry dust has been conducted without DNA extraction. The DNA was retrieved from dust samples by dilution and incubation at 95 °C for 5 min. The direct detection of MDV DNA in the dust by the LAMP assay was possible within 30 min using a water bath and UV light (Woźniakowski and Samorek-Salamonowicz, 2014).

In spite of the apparent advantages of the LAMP assay, it has not gained a great popularity, probably because its intrinsic biotic problematic features. The use of multiple primer pairs at low temperature is not "beneficial" for specificity, as the reaction stringency is low. Moreover, carryover contaminations might occur easily and influence the assay outcome. In addition, the use of multiple primer pairs is feasible only with pathogens were their genomes were fully sequenced.

3.5. Multiplex amplification assays

The multiplex polymerase chain reaction (Multiplex PCR) refers to the use of PCR to amplify several different DNA sequences simultaneously using multiple primers in one tube and one amplification program for all amplicons. By targeting multiple sequences at once, the information is gained from a single test tube and run that otherwise, would require the use of several tubes and several run times, requiring the use of more reagents and a longer time to perform (Elnifro et al., 2000).

Since the first technique description by Chamberlain in 1988 (Chamberlain et al., 1988) many systems have been developed, including assays to amplify simultaneously multiple avian viruses. Unlike other amplification assays, that met wide acceptance, the multiplex assays initiated multiple discussions and occasionally reluctance for acceptance of this technique. The reasons for skepticism was the prerequisite of careful optimization of the amplification reaction components in terms of concentration and annealing temperatures, in order to maintain the optimal specificity and sensitivity for each amplicon. In addition, multiplex amplification assays raised concerns regarding mutual interferences that might be caused due to reagent exhaustion in the reaction tube. For that reason scientists diverged, from enthusiastic developers of multiplexes containing more than two amplicons, to dismissal of the technique. However, at present the PCR reagents are more sophisticated than those that were available in former years. The currently obtainable reagents possess wide dynamic ranges of activity, and the calibration complications seem to be less critical than before. Modern reagents consist majorly of amplification pre-composed mixes, that contain sophisticated amplification enzymes and all components required. Moreover, primers and probes concentrations in the amplification reaction, in general, are in excess (Elfath et al., 2000).

Many studies employed dual multiplex amplifications for two viruses, including our studies that amplified quantitatively the MDV and CAV (Davidson et al., 2013) and ILTV and the fowlpox virus (FPV) from cases of diphteric manifestations in chickens (Fatumbi et al., 1995; Davidson et al., 2015), or two genes of one virus, the TMEV (Davidson et al., 2017b), An originally approach was the development of the multiplex qPCR on the level of two TaqMan probes in one tube to increase the sensitivity of detection of the MDV vaccine and wild type viruses (Davidson et al., 2017c). Several other examples among numerous dual virus amplification assays are (a) the avian metapneumovirus (AMPV) quantitative multiplex subtype A or subtype B to assist in disease diagnosis and to carry out rapid surveillance with high levels of sensitivity and specificity (Cecchinato et al., 2013), (b) Abdelwhab et al. (2010) developed therefore a versatile, sensitive and lineage-specific multiplex RT-qPCR for detection and typing of H5N1 viruses in Egypt.

Multiplex assays including more than two components were extensively developed; for example, Wang et al. (2016) described a multiplex PCR for 6 duck viruses, Zhang et al. (2015) described a multiplex PCR for 11 duck viruses Zeng et al. (2015) for 8 avian immunosuppressive viruses, Li et al. (2016) amplified at once 4 different neuroaminidase types of AIV, subgroup H5N1, Laamiri et al. (2018) described a multiplex assay for four most common avian respiratory viruses, Li et al. (2018) developed an assay for 8 avian influenza A viruses and more. Numerous other examples for multiplex PCRs can be found in the literature, including an assay that incorporated Luminex beads to differentiate the multiplex amplification of AIVs (Laamiri et al., 2016).

The development of such complex multiplex assays seems to provide a fast and economic mean to gain extended information on the presence of several viruses with a one-tube reaction. However, the multiple simultaneous amplifications in one tube might introduce various difficulties, ranging from obvious technical errors, to mutual inhibitory effects that might deteriorate the amplification sensitivities.

3.6. Differentiation between wild-type and vaccine viruses - DIVA

Differentiating Infected from Vaccinated Animals (DIVA) assays were initially developed to identify differentially antibodies to avian influenza viruses and vaccines (Suarez, 2012). The development of recombinant viral-vectored vaccines for the control of avian influenza in poultry is the latest advance in avian virology and vaccinology, because they only express the HA gene. As all the licensed vectored vaccines express only the HA gene, they can potentially be used to differentiate vaccinated from vaccinated and infected birds. Although a potentially valuable tool for the surveillance of the virus in countries that

vaccinate, the DIVA principle has currently not been applied (Suarez and Pantin-Jackwood, 2017).

In view of the extended use of molecular tools for diagnosis, we tried to advance the development of DIVA assays that were based on molecular detection, rather than on serology. However, not many molecular DIVA system were developed for avian viruses by now, therefore that topic turn into a priority for future studies. DIVA assays were developed to provide differential molecular detection assays for vaccine and field viruses of ILTV (Shil et al., 2015). Baigent et al. (2016) described the pp38 MDV DIVA real-time PCR for differential identification of MDV-1 vaccine DNAs from MDV-1 virulent isolate DNAs. That assay was developed using cell-cultured prototype viruses, however, and its employment on feather tip extracts of commercial chickens that have been vaccinated commercially, did not provided the desired differential specificity between the wild-type and the vaccine viruses. For that reason, the simultaneous use of both CVI and Vir3 probes was employed in one tube, as a multiplexed DIVA on the probe level, to provide a new more convenient for use assay (Davidson et al., 2017c). We hypothesized that the competitive binding would provide increased stringency that extend the specificity and the distinctive ability of the DIVA assay. To enable the identification of the two amplifications in one tube, the CVI probe was labeled with a different reporter from the Vir3 probe, the CAL Fluor Gold 540, denoted CVI2 P. was confirmed on commercial samples. Indeed it gained a distinctive capacity between the two viruses (Davidson et al., 2017c). To our knowledge, no Taq-Man qPCR DIVA assays are yet available using multiplexed probes in one tube. Therefore, the present assay contributes originality and additional diagnostic capacity.

In the case of the turkey flavivirus TMEV, the DIVA development (Davidson et al., 2017b) was motivated by the need to differentiate between affected birds as a result of vaccination, or as a result of infection with a TMEV field strain, and relied on whole genomic sequences of the TMEV vaccine and field viruses (Fernández-Pinero et al., 2014).

3.7. Genome sequencing

Thousands of studies describe the inevitable contribution of sequencing techniques for the identification of the infecting viruses in poultry, because classical diagnostic methods, designed to be virus-specific or are limited to groups of viral agents, obstruct the identification of novel viruses or viral variants. Next-generation sequencing (NGS), also known as high-throughput sequencing, is the term used to describe a number of different modern sequencing technologies including: Illumina (Solexa) sequencing, Roche 454 sequencing, Ion torrent, Proton/PGM sequencing and the (Sequencing by Oligonucleotide Ligation and Detection) SOLiD sequencing. NGS is similar to Sanger sequencing as it allows to reveal the sequence of DNA fragments, but in NGS the acquisition of the sequences of all DNA fragments are obtained in parallel. This allows millions of fragments to be sequenced in a single run, as compared to the Sanger sequencing, which only produces one forward and reverse read. These recent technologies allow sequencing DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, and as such, the NGS have revolutionised the study of genomics and molecular biology.

Approaches of NGS provided additional tools for understanding viral diversity and discovery of novel viruses, contributing to provide a better diagnosis and disease control. NGS lead to the identification of a wide range of new viruses and to characterize the viral diversity among avian species. The NGS platforms are being implemented in many clinical and research laboratories, as the costs of these platforms are progressively decreasing (Kapgate et al., 2015).

3.8. Diagnostic methods based on genome sequencing: Pyrosequencing and MinION sequencing

The development of diagnostic assays has advanced by developing in 1993 the pyrosequencing method by Nyren et al. (1993) which combines the benefits of PCR of the DNA sequencing. This assay amplifies a divergent viral fragment that is embraced by two conserved regions on which the amplification primers are designed. Pyrosequencing relies on light detection based on a chain reaction, and then pyrophosphate is released. De Battisti et al. (2013) applied pyrosequencing to diagnose rapidly and to pathotype NDV directly in clinical specimens. NDV is the only member of serotype 1 avian paramyxoviruses (APMV-1) that causes respiratory and neurological disease in chickens and other species of birds and can cause severe economic losses in the poultry sector. Pathotyping is needed for NDV to characterize the infecting viruses. In this study degenerated primers were designed to amplify a portion of the fusion gene responsible for virulence, then a 30-bp region encompassing the cleavage site was pyrosequenced. Pyrosequencing analysis was a useful technique also for genotyping AIV reassortants in the search of vaccine virus candidates (Shcherbik et al., 2014).

The latest development for achieving rapid and affordable molecular diagnosis and molecular epidemiological surveillance was the development of the Oxford Nanopore MinION platform in 2014 (Lu et al., 2016; Leggett and Clark, 2017). The device is exceptionally compact, it is powered from the USB port of a laptop computer, allowing it to be portable outside the dedicated laboratory. The MinION, the first commercial sequencer using nanopore technology, a third-generation sequencing (TGS) technology. The DNA sequencing is performed by measuring the changes in electrical conductivity generated as DNA strands pass through a biological pore. The potential of nanopore sequencing has been demonstrated by various studies in genome surveillance at locations where rapid and reliable sequencing is needed, but where resources are limited. Due to the speed in data production, the MinION is considered suitable for real-time applications.

The MinION third-generation, real-time DNA sequencing platform was introduced also to the detection and characterization of Newcastle disease outbreaks (Butt et al., 2018). The rapid identification and virulence prediction of the circulating NDV are a global challenge to the poultry industry diagnostics, but it is rather complex by the genetic diversity and rapid evolution of NDVs. Spatz et al. (2019) investigated the MinION technology in a phylogenomic approach by examining the the single nucleotide mutations and genomic insertion and deletions in the amplicons generated by the single allele assay. They aimed to develop a fast and affordable genotyping assay for the detection, genotyping and surveillance of emerging ILTV strains in the US, providing by their study a revolutionary horizon in the avian viral diagnosis.

4. Multiple virus co-infections

Multiple virus infections of the same host might have a triple biological significance; (a) Alteration of the clinical and pathological signs, which might differ from those typical to infection with each pathogen in separate, and they usually synergize in the virulence severity. (b) Obstructing the ability to identify one or more of the co-infecting pathogens, (c) Molecular interactions in multiple virus co-infections.

4.1. Alteration of the clinical and pathological signs, due to synergistic infection

The two viruses MDV and CAV cause persistent infections and immunosuppression and synergize in mortality in experimental infection trials and in commercial flocks (Zhang et al., 2017). The pathogenicity of the dual MDV and CAV infection are demonstrated by a variety of symptoms (Haridy et al., 2012) and co-infection of commercial flocks is mostly subclinical, and not acute. We demonstrated the pathogenicity

and the synergism in disease severity caused by the dual infection with MDV and CAV on the enhanced mortality and morbidity, elevated clinical sign score per chick, as well as additional not shown body weight decrease, bursa and thymus atrophy and spleen hyperplasia (Davidson et al., 2019).

AIV-infection could be detected in the single-virus infected group at 4 days post infection (dpi), however it was extended to 6 dpi in the MDV-co-infected chicks and further extended to 8 dpi in the triple-virus infected chicks with MDV and CAV (Davidson et al., 2019). Dual and especially triple virus-infection was correlated previously with changes in the pattern of MDV and CAV horizontal spread (Davidson et al., unpublished), and also with enhanced mortality and morbidity (Davidson et al., 2019). In particular, while AIV H9N2-infected chicks had mild clinical signs, the CAV co-infection caused severe tracheal inflammation.

4.2. Obstructing the ability to identify one or more of the co-infecting pathogens

One of the advantages in the use of PCR to detect oncogenic viruses is its ability to identify sequences of more viruses in the same DNA sample. Isolation of more than one virus in chicken embryo fibroblast (CEF) tissue cultures would not be possible, because the experimental conditions needed to isolate the herpesvirus MDV conflicts those needed to isolate retroviruses (Davidson et al., unpublished). As MDV is a cell-associated virus the CEFs has to be maintained intact for 7–10 days to support virus replication. For the retrovirus isolation, two serial passages in tissue culture are needed, where the first passage has to be frozen in order to free the intracellular virions for re-amplification in the second round of culture. Following that prolonged tissue culturing, the monolayers have to be fixed and immunostained with monoclonal antibodies and inspected for infection. By freezing the first tissue culture passage or the retrovirus isolation, the MDV which might be infected the CEF due to its co-existence in the original sample, would be destroyed as it requires intact cells.

4.3. Molecular interactions in multiple virus co-infections

In cases of multiple virus infection of one cell both viruses can interfere for replication in a single or in a mutual mode, like in cases of certain subgroups of the avian leukosis viruses (ALV) (Fadly and Payne, 2003). Contrariwise, they might not impede each other's replication. In cases that the replication of both viruses are not inhibited, their dual presence in the same cell might lead to their interaction on various levels, either genomic or protein. One of the possibilities of genomic interactions is gene exchanges between the viruses that infect the same cell. The molecular interaction that occurs in multiple virus infections with the five avian oncogenic viruses, that include herpes and retroviruses, and are economically important in veterinary virology consists a biological example for creation of viral diversity through molecular recombination (Davidson and Silva, 2008; Davidson, 2009a). Genomic exchanges between viruses can occur between two RNA viruses, between two DNA viruses, or between DNA and RNA viruses.

These inter-species and intra-species molecular recombination events are one of the mechanisms that contribute to the creation of diversity in animal viruses (Davidson and Silva, 2008). Many mechanisms might contribute to the variety of life on earth, such as shuffling of genetic material between the host and the infecting virus and between viruses belonging to the same or a different family, or between viruses infecting various hosts.

Chickens have a distinct advantage for the study of virus diversity, because they are the natural hosts for the infecting viruses. In addition, no separate experimental models are needed to be performed for the disease reproduction and no infections with various pathogens are needed. Many natural infections of chickens can be considered, therefore, as reflecting real and natural situations and not artifactual events. We studied avian viruses because they are economically important for agriculture, but also suitable for reflecting *in vivo* recombination events that might shed knowledge on general virology (Davidson and Silva, 2008). Moreover, virological studies on poultry have additional advantages because of their relevance to "real life" virology, because chickens in commercial poultry houses are exposed to environmental conditions of stress that might influence the disease outcome. Also, large number of birds in commercial flocks enables study of low-rate events.

The likelihood that viruses can interact molecularly in multiple virus infections and might change their molecular configuration accentuate the importance of being aware to perform molecular diagnosis directly on sampled organs, and not after further amplification in *ex vivo* systems, to avoid artifactual results.

4.3.1. Molecular interactions between two RNA viruses

Among the avian RNA viruses, two retroviruses can recombine either *in vitro* or *in vivo* in cases of intentional or natural dual virus infections. The most outstanding example for *in vivo* recombination, led to the creation of the new avian leukosis virus, subgroup J (ALV-J). ALV-J emerged following a spontaneous recombination between exogenous and endogenous retroviral sequences (Bai et al., 1995; Venugopal, 1999). Very soon the new virus disseminated worldwide with the extensive international trade of the specific genetic breed, in which ALV-J was created, leading to a very severe outcome with a devastating economic impact on the poultry industry (Malkinson et al., 2004). ALV-J genetic sequence revealed several recombinations between the exogenous ALV gag and pol genes and the *env* gene of the endogenous avian erythoblastosis virus (Bai et al., 1995; Benson et al., 1998; Fadly and Smith, 1999). In the respect of viral evolution through genetic recombination, ALV-J represents a viable recombinant that occurred spontaneously *in vivo* between exogenous and endogenous avian retroviruses, ALV and AEV, respectively. While the LTR, gag and pol genes are highly homologous with other ALV subgroups, the *env* gene had only 40% identity with other exogenous ALV *envs*, but 75–95% homology with *env*-like genes of the endogenous avian retroviruses (EAV) family (Fadly and Payne, 2003). A number of other *in vivo* recombination events between ALVs have been described; an ALV-J encoding an ALV-A envelope (Lupiani et al., 2003, 2006), an acutely transforming isolate of ALV-J (Chesters et al., 2001), a recombinant ALV containing the ALV-J sequence uncovered examples of *in vivo* recombination events between RNA viruses which commonly infect same lymphocytes in the chicken, as lately documented for the Australian breeding flocks which were co-infected with ALV-A and ALV-J (Bagust et al., 2004; Fenton et al., 2005).

4.3.2. Molecular interactions between MDV and retroviruses

The retrovirus recombination process with MDV occurs because retroviruses integrate into any double stranded DNA genomes for replication; in a MDV-infected cell, the integration can occur into the cellular or into the MDV genome. The documented inserts of avian retroviral sequences, were mainly the LTR, and those were gathered at the junctions between the unique (long or short) MDV fragments and the terminal or internal repeated MDV fragments (TR_L and TR_S and IR_L and IR_S) (reviewed in Brunovskis and Kung, 1996). Integration of the retroviral sequences into the herpesvirus genome was documented *in vitro* by co-infecting CEF cultures with MDV and the retroviruses REV and ALV (Brunovskis and Kung, 1996). By co-cultivating MDV and REV in the same tissue culture dish the first recombinant virus, RM1, was created. RM1 was characterized both molecularly and biologically as having altered *in vitro* and *in vivo* biological properties.

Having experienced the relatively efficient creation of recombinant viruses *in vitro*, we questioned whether retrovirus integrates into DNA viruses also *in vivo*, in the bird, in multiple viral infections. If such process would occur, serious consequences might follow; recombinant MDV might possess altered biological properties, and relatively known

features of these viruses could turn into unknown and unpredictable patterns. Putative features whose changes might be biological significant are, pathogenicity, virus spread, antigenicity and immunogenicity leading to changes in the ability of specific vaccines to protect against diseases. In our studies on *in vivo* recombination events we analysed the integration events within the original organs, and not in viruses that were re-cultured *in vitro*, in order not include artifacts, as frequent genetic changes occur upon *in vitro* virus-replication processes (Davidson, 2009b).

The further replication of the viruses which contained chimeric molecules in cell cultures, process which might have increased the viral amounts, was strictly avoided (Davidson and Borenshtain, 2001; Borensthain et al., 2003). For that reason several experimental difficulties were met compared to the studies performed in tissue cultures. However, in spite of all difficulties, we showed that retroviruses could integrate into the MDV genome as exemplified by the detection of chimeric molecules, directly within the DNA that was purified from the tumor-bearing chicken (Davidson and Borenshtain, 2001; Borenshtain et al., 2003).

Unlike *in vitro*, where recombinant viruses were separated by several rounds of plaque purifications and limiting dilutions, the *in vivo* situation differs; many different events occurred simultaneously in the same bird as each cell produces many herpes virions. As various molecules were formed and detected in the same DNA preparation, recombinant virus isolation was problematic. Only a biological advantage would enable a recombinant virus to dominate in an infected bird. In addition, the *in vivo* herpes-retro recombination issue differs, and is rather more complex than *in vitro* co-cultivation of the two virus types; the cells in the *in vitro* co-infection were fibroblasts, whereas *in vivo*, the target cells are mostly lymphocytes and monocytes. While the recombination rate *in vitro* was rather high, the *in vivo* formation of viable recombinant viruses depends on the presence of host immune responses, tissue affinities, and more, therefore, the amounts of recombinant viruses is often minute. We concluded therefore, that both situations cannot be extrapolated, although showing that commercial poultry co-infections have the potential for a collective clinical influence and also can result in the emergence of recombinant viruses, possibly with unexpected biological properties (Davidson and Silva, 2008).

The issue of retroviral sequence integration into herpesviruses *in vivo,* in cases of double virus-infection is of a wide significance in general virology, veterinary medicine and also represents a special case of gene transposition. We determined the occurrence of such integrations *in vivo* by following the presence of chimeric molecules. Several conditions were analysed: a) Commercial birds that acquired naturally a mixed infection; b) Experimentally infected chickens with prototype strains of MDV and ALV-J; c) Commercial chickens infected experimentally with virus inoculae obtained from commercial cases of double infection with MDV and ALV-J, in the same flock or the same bird. In the two first categories we found that integration events happened at various rates, depending on the experimental system used. While in commercial flocks the event was limited (about 2.5% of the 2926 DNA samples), it reached a 30–50% rate in experimentally-infected birds with prototype viruses, and was undetectable in experimentally-infected birds with field inoculae. It seemed that by increasing the virus adaptation to laboratory conditions, the rate of retrovirus LTR integration into MDV increases, as judged by the extent of chimeric molecules. Also, in each DNA preparation a variety of chimeric molecule types were detected, indicating the *in vivo* formation of molecular quasispecies in dually-infected birds.

The chimeric molecule heterogeneity found now might indicate that several integrations occurred in one double virus-infected cell or reflects the events in several cells, as each DNA preparation originated from numerous cells. As such, each DNA sample might differ in the molecular population content.

Molecular intergrations of avian retroviruses in avian DNA viruses occurred also between the FPV (Tripathy and Reed, 2008) and REV. Kim and Tripathy (2001) analysed early isolates of FPV and showed that the event of REV integration into FPV occurred more than 50 years ago. However, REV envelope sequences have been detected only in FPV field strains, suggesting the presence of intact REV genome in wild-type strains. Unlike MDV, where the retroviral-LTR integrations occurred at various genetic sited within the junctions of the unique and repeat MDV genomic fragments, the retroviral-LTR integrations occur within the FPV genome at a specific site, between open reading frame 201 and 203 (Brunovskis and Kung, 1986; Garcia et al., 2003). Up to this point it is not elucidated yet whether the emergence of new FPV isolates, the disease re-emergence and the apparent lack of vaccine efficacy are due to REV insertions. We havedemonstrated that most, but not all the FPV isolates in commercial flocks contained REV inserts of various sizes (2008c).

4.3.3. Molecular interactions between DNA viruses

Multiple viral infections of chickens with DNA viruses are probably the ground on which genetic exchanges between these viruses occur. To our knowledge, two studies documented natural dual infections of chickens, employing FPV (Thipathy and Reed, 2008), FPV and Infectious laryngotracheitis (ILTV) virus (Fatunmbi et al., 1995) and FPV and MDV (Tripathy et al., 1975). These events might facilitate, in a yet unknown mechanism, transfer of genomic fragments between DNA viruses. Although the rate of these DNA movements are supposed to be even lower than events which involve RNA viruses, Brunovskis and Velicer (1995) provided evidences that several FPV genes have homologs in the MDV genome.

5. Final consideration

The diagnosis of veterinary viruses is substantiated by two pillars side by side, the clinical signs and the laboratory diagnosis. Neither of these two components can stand alone, as clinical diagnosis is not definitive without laboratory confirmation, while the latter is insignificant without at least one virus-specific clinical sign. Moreover, diagnosis based on macroscopic lesions might be heavily influenced by subjectivity, and might be indistinctive and insensitive, signs might be common to several diseases. Also, molecular diagnosis of virus infections virus enable the diagnosis even in absence of clinical signs or postmortem lesions. Nowadays, revolutionary technological developments contributed to creation of novel sophisticated diagnostic assays that are fast, sensitive and highly informative. However, a special attention should still be dedicated to the biotic factors in diagnosis, which might substantiate the correct diagnosis by performing the accurate sampling and approach in view of the pathogen and disease biology.

Furthermore, with the availability of such powerful diagnostic means, it is questionable whether to employ classical virological procedures besides the molecular diagnosis. The response is positive, it is still needed to collect field viruses, to perform amplify those samples in embryonated eggs or in tissue cultures to obtain purified stocks of live viruses for future reference, and for use as the challenge in experimental infection trials. These experimental infection trials might be performed in search of the protection ability of new vaccines, or other future biological research. Furthermore, to determine the presence of live viruses virus replication has to be demonstrated. In these cases the molecular diagnosis is not the ultimate diagnostic mean, because molecular assays can also perform on viral nucleic acids that originate from inactivated or non-replicable viruses. Thus, classical virology toolbox receives considerable enhancement by technological innovations rather than being replaced by them.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

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