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REVIEW

Classification of Marek's disease viruses according to pathotype: philosophy and methodology

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The concept of pathotype in Marek's disease (MD) probably dates from the recognition of a more virulent form of the disease in the late 1950s (Benton & Cover, 1957). Distinctions between MD virus strains were further expanded with the description of the vv pathotype in the early 1980s and of the vv + pathotype in the 1990s. Pathotype designations reflect important biological properties that correlate with the break-through of vaccinal immunity in the field. However, pathotyping methods applied by various laboratories have not been uniform, preventing critical comparison of results. Better uniformity of pathotyping procedures is desirable.

The Avian Disease and Oncology Laboratory (ADOL) method is based on induction of lymphoproliferative lesions in vaccinated chickens. This method has been used to pathotype more than 45 isolates and is the basis for the current pathotype classification of MD virus strains. Its limitations include requirements for a specific type of chickens (15×7 ab+), large numbers of animals, and a statistical method to compare lesion responses to those of JM/102W and Md5 control strains. Because of these limitations, it has not been and is not likely to be used in other laboratories.

Comparability in pathotyping can be improved by the comparison of field isolates with standard prototype strains such as JM/102W, Md5 and 648A (American Type Culture Collection) or their equivalents. Data may be generated by different *in vivo* procedures that measure tumour induction, neurological disease (both neoplastic and non-neoplastic lesions), or solely non-neoplastic criteria (such as lymphoid organ weights or virus replication). Methods based on neoplastic criteria, especially when generated in MD-immunized chickens, will probably correlate most closely with that of the ADOL method and be most relevant to evolution of MD virus in the field. Based on data from several trials, a modification of the ADOL method that utilizes fewer chickens and can be conducted with commercial specific pathogen free strains is proposed. The modified method is based on "best fit" comparisons with prototype strains, and is expected to provide results generally comparable with the original method. A variety of other alternative criteria (see earlier) are also evaluated both for primary pathotyping and as adjuncts to other pathotyping methods. Advantages and disadvantages of alternative methods are presented.

Introduction and History

Virulence is a property associated with those Marek's disease viruses (MDVs) designated as serotype 1. Related herpesviruses of serotypes 2 and 3 are considered non-oncogenic. Virulence is usually measured in terms of the ability to induce lymphoproliferative lesions in chickens, normally characterized by enlargements of peripheral nerves due to lymphoid infiltration and lymphomas in various visceral organs or tissues. Virulence is important for many reasons, but especially because this property varies among serotype 1 MDV isolates and is directly related to the ability of isolates to be protected by vaccines. Also, virulence of MDV strains has increased over the years (Witter, 1997), a trend that continues to

the present time and represents a formidable obstacle to the long-term control of the disease.

The term "pathotype", although properly used to designate classes of organisms that induce different types of pathology, has in the case of Marek's disease (MD) been widely applied to designate differences in the virulence of isolates as measured (primarily) by the frequency of disease induced. This reflects the practical importance of virulence, especially the ability to cause disease in immunized chickens, compared with other classification criteria. The use of "neuropathotype" to designate strains that induce distinctly different types of pathology in the central nervous system (Gimeno *et al.*,

2002) is a more conventional application of pathotype terminology.

Soon after the development of reliable methods for its biological transmission (Sevoian *et al.*, 1962; Biggs & Payne, 1963), variation in the virulence of MD strains became apparent. In what is probably the seminal observation, Biggs and coworkers reported that the newly isolated HPRS-16 and HPRS-18 strains induced higher mortality with shorter latent periods and more visceral lymphomas compared with the previously isolated B14 strain (Biggs *et al.*, 1965). These authors proposed that strains be classified as "classical" or "acute", with the acute strains differentiated by the induction of higher morbidity and mortality, earlier onset, and a higher incidence of visceral tumours compared with classical strains. Interestingly, this first proposal for what was essentially a pathotypic classification for MDV preceded by several years the isolation and identification of the aetiological agent of the disease. Use of this classification to type additional field isolates (Purchase & Biggs, 1967) reflected correctly a significant shift of virulence in MD that would be identified again in later classifications.

A set of criteria for differentiating between acute and classical strains on the basis of histological lesions in nerves and viscera was later described (Biggs & Milne, 1972). Acute isolates had higher lesion scores (>1.5) on a 1 to 5 scale than classical isolates (<1.5). Thus, classification was a product of both virulence and tissue tropism. Strains such as JM and GA differ greatly in tissue tropism but are equally virulent (Purchase *et al.*, 1971) and equally protected by herpesvirus of turkeys (HVT) vaccination: both are properly classified as acute strains. This suggests that virulence may be more reliable than tissue tropism as a criterion for classification, although the two parameters are often linked.

Important variation in virulence and induction of humoral immune responses was observed between the JM-10 and CU-2 isolates (Smith & Calnek, 1973) but no unique nomenclature was proposed, even though subsequent work places these isolates in different pathotypes.

A shift of virulence among MDV strains recognized in the late 1970s in association with an unexplained increase in MD losses in vaccinated flocks (Eidson *et al.*, 1978) prompted further evolution in the process of pathotype identification. Witter *et al.* (1980) isolated and characterized several strains from Maryland that caused a higher frequency of MD lesions in HVT-vaccinated chickens than earlier isolates. These isolates were originally considered as "variant" viruses but later were termed *very virulent* or *vvMDV* (Witter, 1983) as a more appropriate way to distinguish these isolates from earlier *virulent* or *vMDV* strains. The initial method was based on the arbitrary criterion of HVT protection— isolates against which HVT provided 77% protection or less were considered as vv (Witter, 1983). Some laboratories applied vv nomenclature if the isolate caused significant disease in vaccinated chickens (Powell & Lombardini, 1986; Jurajda & Halouzka, 1988; Mckimm-Breschkin *et al.*, 1990). In some cases, an isolate was termed "vv" pathotype for no reason other than it was derived from vaccinated flocks with excessive MD losses (Witter, 1988). In response, Witter modified the definition to the following: "vvMDV isolates are those which induce MD lesions in HVT-vaccinated,

susceptible chickens at a rate greater than that of a prototype vMDV isolate, such as JM or GA" (Witter, 1988). This helped to establish the important concept of pathotype determination based on comparison with a prototype strain.

This concept was soon adopted by several laboratories. Imai and coworkers (Imai & Yuasa, 1988; Imai *et al.*, 1992) designated isolates as vv by multiple criteria, including a comparison with prototype vv strains Md5 and RB1B. Buscaglia and coworkers typed isolates using P2a chickens in Argentina by comparison with control strains either from sister trials conducted in the United States (Buscaglia *et al.*, 1995) or, later, with direct comparisons with the JM and RB1B strains (Buscaglia *et al.*, 2004). Liu *et al.* (1996) typed isolates as vv by comparison with the control strain GA. The C12/130 strain was identified as having special virulence properties by comparison with the prototype strain HPRS-16 (Venugopal *et al.*, 1996), although a pathotype designation was not applied in this work. Sung classified the KOMD-IC isolate as vv pathotype on the basis of comparisons with the JM strain (Sung, 2002). On the other hand, other reports have described what appear to be highly virulent isolates without benefit of a prototype virus control (Kross, 1996; Lin & Chen, 1996). The preceding authors were all working to characterize new MDV isolates and, in some cases, to assign pathotype designations. However, the procedures varied widely.

The driving force behind a pathotypic classification was the association of pathotype with the field disease. Each new pathotype appeared associated with a new wave of MD losses in commercial chicken flocks. Also, each pathotype appeared associated with the ability to be protected by specific MD vaccines. For example, v pathotype strains induced high levels of disease in non-vaccinated chickens, but little disease in chickens vaccinated with HVT. In contrast, vv pathotype strains induced high levels of disease in HVT-vaccinated chickens, but little disease in chickens vaccinated with bivalent vaccines composed of HVT and selected serotype 2 strains such as SB-1 or 301B/1.

Thus, four pathotypes of serotype 1 MDV are currently recognized; m (mild), v, vv and vv+ (Witter, 1997). Pathotype m includes strains previously termed as classical. However, no m pathotype strains have been recognized among recent isolates and the frequency of this pathotype in the field is uncertain.

Yet another wave of increased MD losses in vaccinated flocks was noted in the early 1990s, especially in the United States. Some of the isolates from such flocks caused higher frequencies of MD in bivalent-vaccinated chickens than did prototype vv pathotype strains such as Md5. Witter proposed that these strains may represent yet a new pathotype and proposed the designation "vv+" (Witter, 1997). Once again, a new pathotype was associated with a change in field disease incidence and the failure of established vaccines to provide adequate protection.

The present Avian Disease and Oncology Laboratory (ADOL) method for determination of pathotype (Witter, 1997), in use since approximately 1989, is based on the induction of lymphoproliferative lesions in vaccinated chickens (see later description). This method has been used to pathotype more than 45 isolates and can probably be considered as the gold standard for pathotype classification of MDV strains. However, the re-

quirement for a specific type of chicken (15 ×7 ab+) and the high cost of keeping large numbers of chickens through a 9-week experimental period have deterred use of this method by other laboratories. Consequently, isolates continue to be described where pathotyping data are insufficient to permit critical evaluation.

This problem is no doubt responsible for the recent introduction of the term "hypervirulent", which has been used to designate serotype 1 field isolates (such as the Italian isolate, EU1, isolated in 1992) that induce tumours in CVI988-vaccinated chickens (Schumacher *et al.*, 2002; Burgess, 2004). Such isolates had sufficient virulence to warrant a unique designation but could not be identified as vv+ because formal pathotyping by the ADOL method was not available in European laboratories. Hypervirulent and vv+ designations should not be considered to be equivalent because no definitive criteria for classification of viruses as hypervirulent are available.

MD isolates that differ in ability to induce MD lesions in vaccinated chickens also tend to differ for additional biological characteristics. Compared with less virulent isolates, the more virulent isolates are generally considered to produce more visceral tumours with an earlier onset of tumour mortality, to cause disease in genetically resistant strains of chickens, to cause more immunodepression often associated with severe lytic changes in lymphoid organs, to induce early non-neoplastic death (early mortality syndrome), to induce more transient paralysis, and to replicate more rapidly and to higher titres *in vivo*. Some additional characteristics such as severe lytic infection (Kross, 1996; Venugopal *et al.*, 1996), severe proliferative lesions in the brain (Cho *et al.*, 1998), haemolytic anaemia (Gilka & Spencer, 1995), and tropism for monocytes (Barrow *et al.*, 2003) have been associated with certain highly virulent strains, but are not necessarily associated with any of the designated pathotypes. Several of these characteristics have been proposed as alternative criteria for pathotyping.

In confirmation of earlier studies (Rivas & Fabricant, 1988), Calnek *et al.* (1998) observed a correlation between pathotype and characteristics related to immunodepression; that is, persistence of early cytolytic infection and atrophy of thymus and bursa of Fabricius—these workers proposed that measurement of lymphoid organ weights might be useful for classification of MDV isolates by pathotype. Sung (2002) utilized bursal weight data in the pathotypic classification of a field isolate.

Based on the earlier observation that highly virulent MDV strains induced an acute form of transient paralysis (Witter *et al.*, 1999), Gimeno *et al.* (2002) proposed a classification of MDV strains into three neuropathotypes. Although the neuropathotypes were correlated with virulence and pathotype, the relationships were not perfect and this classification was considered an adjunct to rather than a replacement for conventional pathotyping assays (Gimeno *et al.*, 2002).

It is widely understood that highly virulent MDV strains replicate earlier, faster and induce higher viraemia titres *in vivo* than strains of lower virulence (Rosenberger, 1995). However, there have been only a few published studies that clearly document this relationship (see later discussion). Quantitative polymerase chain reaction (PCR) assays should be useful to establish the amount of viral genomic DNA present after infec-

tion with isolates of differing virulence, but little data have yet been published. Thus, viral replication rates and viral load can also be considered as another possible criterion for pathotyping.

The absence of a universal pathotyping method that can be applied in many laboratories around the world is a serious limitation to progress on MD research. The primary objectives of this review are to discuss the concept of pathotypic differentiation of MDV strains and to summarize the existing technology applicable to the classification of strains according to pathotype. Some previously unpublished data relevant to this discussion are included. A modified pathotyping assay based on comparison of test virus responses with that of prototype virus controls is proposed that provides results generally consistent with the ADOL method and presumably could be conducted by reference laboratories. A secondary goal is to discuss the merits of different response criteria, including immunodepression, clinical neurological signs, and virus load as determined by quantitative PCR. The intent of this review, therefore, is to stimulate movement towards greater uniformity in pathotyping technology. With uniform methods, it should be easier to compare data between laboratories and obtain agreement on pathotypic classification of isolates. In turn, this will provide a better basis for the identification of biological and molecular properties that are relevant to virulence.

Why Pathotyping?

Determination of pathotype is useful for a number of reasons. Probably the most frequent application is to investigate the cause of excessive MD losses in vaccinated flocks. Producers often assume that such losses indicate the presence of exceptionally virulent strains, although this is not always the case. Control strategies for MD are much the same regardless of the pathotype of virus present. Another application is the detection of new pathotypes, and to document further evolution of MD viral strains to greater virulence. If many strains are evaluated, information may be obtained on the relative proportion of the isolatable virus population represented by each pathotype. A battery of properly pathotyped virus strains is the starting point for research to associate molecular properties of MD viruses with virulence. Another important reason is to obtain the most recent and most virulent strains to use as challenge viruses to test the protective efficacy of vaccines. Thus, pathotyping will continue to be a valuable tool in advancing knowledge on MD. An elegant, early example of pathotyping as an epidemiological tool is provided by Biggs *et al.* (1972), who found different pathotypes of MD virus present in different chickens reared on the same farm.

The Virus Stock is Important!

Viruses to be pathotyped, or to be used as prototype controls in pathotyping assays, should meet a number of criteria. In all cases, the stock should be free from contaminants, have sufficient titre, and be available as a collection of similar ampules held as a virus stock (so that replicate assays could be conducted). It should not be passed excessively in cell culture, as evidence of attenuation has been observed between 20 and 30 passages. A seed stock system is recommended to keep

passage levels consistent and low. Most stocks are stored as cryopreserved suspensions of infected cells at $-196°C$. Several of these issues are critical and are discussed later.

Virus stocks may be prepared as low-passage cell culture suspensions or as suspensions of lymphocytes from the blood or spleen. Advantages of cell cultures include easier titration by plaque assay, ability to develop high titre stocks that contain $\geq 10^6$ plaque forming units (PFUs) per millilitre, and ability to screen out chicken anaemia virus (CAV), a common contaminant that does not replicate in most avian cell cultures. We have found that after six passages in duck embryo fibroblasts (DEF), CAV can no longer be detected (Witter, unpublished data). Chicken kidney cultures are also commonly used for MDV isolation and propagation (Churchill & Biggs, 1967; Schat, 2005). Advantages of lymphocyte stocks are principally to avoid possible attenuation or other mutations that might occur during cell culture passage. It is important to keep the type of stock consistent in a given experiment; cell culture and lymphocyte stocks should not be compared in the same set of assays.

Only pure stocks of serotype 1 MDV isolates should be pathotyped. Inadvertent contamination of MD viral stocks with CAV, reticuloendotheliosis virus, avian leukosis virus, or other agents has been common in the past and may seriously bias results of pathotyping assays. CAV contamination has been mainly a problem with lymphocyte stocks (Miles *et al.*, 2001) since the virus does not propagate in most cell cultures, but all stocks need to be tested, preferably by nested PCR assays. Reticuloendotheliosis virus and avian leukosis viruses propagate well, without cytopathic effects, in the same cultures used for propagation of MDV and are detected by the usual assays. Since MD vaccine viruses persist for life in vaccinated chickens and commonly are present in samples used to isolate field strains (De Laney *et al.*, 1995), it is also necessary to insure that the virus stock is free from vaccine strains or serotype 2 strains present as natural infections in field flocks. This is best done by immunofluorescent assays using serotype-specific monoclonal antibodies (Lee *et al.*, 1983; Cui *et al.*, 2004). MD isolates that stain with antibody H19 (serotype 1) but are negative with antibody Y5 (serotype 2) or L78 (serotype 3) are assumed to be pure serotype 1. However, if the isolate was obtained from chickens vaccinated with CVI988 (which is not stained by antibody H19), one may also stain with antibody T65 that detects the CVI988 and GA strains but not other serotype 1 viruses (L. F. Lee, personal communication; Lee *et al.*, 1983; Cui *et al.*, 2004). Such immunofluorescent assays should be applied to cultures that contain >100 plaques, since the number of plaques examined determines the sensitivity of the test. Cloning or plaque purification at the initial isolation step will help reduce problems from vaccine strain contamination. Also, isolation from chickens with gross tumours usually insures a favourable ratio of virulent virus to avirulent vaccine strains.

The number of cell culture passages is important. The number should be kept low and consistent. At ADOL, master seed stocks of field isolates are prepared at DEF passage 6. Working stocks are prepared at passage 7 or 8. Since isolates show little or no change in biological properties between passages 6 and 20 (Witter, unpub-lished data), we assume there is little or no attenuation during the initial six to eight passages. However, this is impossible to confirm.

Cloning deserves special consideration. A cloned virus is by definition derived from a single virus particle. In the MD system this is usually achieved by inoculation of cell free virus obtained from feather follicles or sonicated cell cultures and propagating a virus stock from a single plaque. Its purpose is to achieve a uniform virus population and to exclude contaminants, important attributes of any virus used in research. In practice, however, a cloned MD virus may quickly become heterogeneous upon cell culture passage. Furthermore, a cloned virus may not be as representative of a virus population on a farm as an uncloned virus. In contrast, plaque purification is usually achieved by inoculation of cell associated virus and propagating a virus stock from a single plaque. Plaque purification is used to reduce the likelihood of contamination with other MD serotypes but should not be considered a substitute for cloning. Whether to clone or plaque purify is an important decision, which is only partly dependent on the requirement of pathotyping assays.

A final caution is that the same MD viral strain used in different laboratories or prepared at different times under different conditions should not be assumed to be identical. Isolate 615K was isolated at ADOL from material provided by J. Rosenberger identified as strain T. King, but it is unlikely that the cell culture propagated 615K and the T. King isolate propagated in chickens by Rosenberger will have identical properties. A preparation of strain RB1B, obtained from Cornell University, proved to be significantly less virulent after propagation at ADOL than its parent strain (Gimeno *et al.*, 1999). Unfortunately, this problem is very difficult to identify since there is rarely the opportunity for side-by-side evaluation of a particular isolate and its original parent strain. This issue also exists for viruses cloned from a characterized parent strain.

The Chicken is Important Too!

Virulence is defined by the frequency and severity of disease induced in a susceptible host chicken. Chickens vary greatly in MD susceptibility due to genetics, maternal antibodies, and vaccination status. No single type of chicken will adequately differentiate all the various degrees of virulence represented by different MDV isolates. Thus, it is desirable to use multiple groups of chickens with graded levels of susceptibility. Highly susceptible chickens should differentiate the least virulent strains whereas highly resistant chickens will better differentiate strains with higher levels of virulence.

Creation of a suitable series of chicken types with differential susceptible to MD is a critical step for pathotyping assays. This can probably be done in several ways. For the ADOL assay, a highly susceptible chicken (cross 15×7) derived from vaccinated parents was used. Non-vaccinated, HVT-vaccinated, and bivalent (serotype $2 + 3$)-vaccinated groups were used to provide three groups with graded levels of susceptibility. Vaccination was considered a better choice than selection of lines with different genetic susceptibility because of the relevance of vaccines to the practical control of the disease. Other genotypes of chickens should also be useful, as discussed subsequently. If the chicken strain

has moderate levels of genetic resistance, less disease may be induced but it still should be possible to differentiate pathotypes using non-vaccinated and vaccinated groups.

Homogeneity of response is also important. Crosses of inbred lines such as 15 × 7 would be expected to yield more homogeneous responses than would non-inbred stock. It may be useful to consider the genetic diversity, especially at the major histocompatibility complex, and the degree of inbreeding when selecting a chicken stock for pathotyping studies. Freedom from extraneous infections is critical. Chicks from specific pathogen free (SPF) breeders are desirable, although if MD maternal antibodies are desired to decrease MD susceptibility, MD-vaccinated SPF breeders may be utilized.

The ADOL Pathotyping Assay (Gold Standard)

Before addressing other options, it may be useful to review the current ADOL assay (Witter, 1997) and its applications to pathotyping. The data output is based on the induction of lymphoproliferative (tumour) lesions. The data are compared with that of prototype strains. The design tests for the ability of a test virus to break through immunity induced by two classes of standard, commercial vaccines. Each of these features helps insure that the typing is consistent with classified strains and is relevant to the process of MDV evolution.

The assay is performed by simultaneous challenge of three classes of 15 × 7 ab+ chickens (from dams immunized with all three MDV serotypes); unvaccinated, HVT-vaccinated and HVT+SB-1-vaccinated. Vaccines are administered at hatch at 2000 PFU per chick. Challenge is performed at 5 days post vaccination using 500 PFU per chicken. Two prototype strains, JM/102W (v pathotype) and Md5 (vv pathotype), are used as controls. Each lot consists of 17 chickens that are housed in modified Horsfall–Bauer isolators to prevent inadvertent spread of infection between treatment groups. The test is terminated and all chickens necropsied at about 56 days post challenge. The experiment is fully replicated, usually at a subsequent date. Thus, each virus is classified on the basis of responses from 102 chickens (three treatments x two replications). The gross lesion response data of test strains are compared with those of control strains using an interaction chi-square analysis (Steel & Torrie, 1960). Pathotypes are designated on the basis of total lesion responses according to the following:

v   Response in HVT-vaccinated chickens does not differ from JM/102W.
vv   Response in HVT-vaccinated chickens exceeds that induced by JM/102W and does not differ from that induced by Md5 in HVT/SB-1-vaccinated chickens.
vv+ Response in HVT/SB-1-vaccinated chickens exceeds that induced by Md5.

Another useful parameter, designated as *virulence rank*, can be calculated from the same response data (Witter, 1997). The calculation is 100 minus the mean of the protective indices obtained in HVT-vaccinated and HVT/SB-1-vaccinated chickens. The virulence rank permits a convenient numerical ranking of test isolates from 0 (very low virulence as indicated by high protection by both vaccine types) to 100 (very high virulence as indicated by low levels of protection by both vaccine

types). Since this parameter is based on response data from four lots of chickens representing those vaccinated with HVT and with HVT/SB-1, respectively, the parameter reflects well the ability of a virus to break through two different vaccine types.

Although the ADOL assay is generally reproducible, there are a number of limitations. The ADOL 15 × 7 ab+ chickens are not readily available for use by other laboratories. The power of the statistical assay depends on a standard number of chickens and replicates, which limits the ability to adjust group sizes and replicate numbers. The 9-week holding period results in a very high cost for chicken maintenance. Each treatment (three chicken types x two replications) requires 102 chickens. Thus the number of chickens required to type 2 test viruses (using two prototype control strains) is over 400 chickens. Also, the assay is not designed to differentiate m pathotype strains, although it could be adapted to do so (see subsequent section).

Reproducibility is relative. We have noted substantial variation between replicate trials. For example, virulence rank data for the JM/102W and Md5 strains over 10 replicate trials conducted under nearly identical conditions but at different times were 8.0 to 32.2 (mean 17.6) and 39.5 to 69.0 (mean 56.3), respectively (Witter, unpublished data). Similar variation among replicate control data has been reported earlier (Witter, 1997).

To exemplify the utility and application of the procedure, data from ADOL assays on six new field isolates are presented in Table 1. In this case, all six isolates induced very high rates of MD in non-vaccinated chickens. The incidence of MD in HVT-vaccinated chickens varied from 45% to 100%, levels significantly greater in all cases than those induced by the JM/102W strain. This indicated that the strains were vv pathotype or greater. The incidence of MD induction in bivalent-vaccinated (HVT+SB-1) chickens varied from 3% to 58%. The MD frequency of isolate 686 was significantly greater than the Md5 strain control, indicating that this strain was vv+ pathotype. The other five strains did not differ from the Md5 control and were thus designated as vv pathotype. One should note, however, the variation in MD response induced by Md5 in trials 1 and 2, and the differences between both of these values and the mean of values from 10 previous trials. Depending on which value for Md5 was used for comparison, the pathotype designations would differ. This illustrates one type of problem in the application of the standard ADOL assay.

Modifications of the ADOL Assay

We have investigated two different approaches to improve the standard ADOL assay: (1) inclusion of CVI988-vaccinated chickens in the challenge model, and (2) use of alternative strains of chickens (and different response criteria).

CVI988-vaccinated chickens. Recent studies have addressed whether the use of CVI988-vaccinated 15 × 7 ab+ chickens would be a useful adjunct to the standard ADOL method. Groups of CVI988-vaccinated chickens were challenged with a panel of seven vv and seven vv+ strains, and the results compared with those from bivalent-vaccinated (HVT+SB-1) chickens in earlier trials (Table 2). The mean per cent MD lesions and the per cent protection between the two groups did not

Table 1. *Pathotyping of six new isolates by the ADOL assay[a]*

Trial	Isolate	% MD in chickens vaccinated with:			Pathotype	Virulence rank
		None	HVT	HVT+SB-1		
1	670	100.0	45.2*	3.1	vv	24.1
	685	100.0	52.9*	6.3	vv	29.6
	686	100.0	100.0*	57.6*	vv+	78.8
	JM/102W	100.0	5.9			
	Md5	100.0		14.7		
2	690	100.0	63.6*	39.4	vv	51.5
	691	97.1	52.9*	5.9	vv	30.3
	692	100.0	97.1*	50.0	vv	73.5
	JM/102W	84.8	19.4			
	Md5	100.0		44.1		
Historic controls	JM/102W	92.8	22.3	8.7		
	Md5	99.8	82.8	28.0		

[a]The standard ADOL pathotyping assay (Witter, 1997) was applied to the typing of six new field isolates (trials 1 and 2). Line 15×7 chickens were the F1 progeny of line $15I_5$ males and line 7_1 females. The dams received MD vaccine of all three serotypes (Witter, 1987) so that progeny chicks were considered positive for maternal antibodies (ab+). Groups of 17 chickens were vaccinated at hatch with 2000 PFU of the respective vaccine (none, HVT or HVT+SB-1), challenged with 500 PFU of the respective challenge virus at 5 days post vaccination, and held in modified Horsfall–Bauer isolators under negative air pressure until the conclusion of the trial, 8 weeks post challenge. The per cent (%) MD responses are based on the number of dead or killed chickens with gross MD lesions; data are pooled from two replicate trials. Virulence rank was calculated as 100 − mean % MD response (HVT and HVT+SB-1 groups). The table also includes mean response data from 10 earlier trials (Witter, 1997) for comparative purposes. * Response values greater than that for the appropriate control virus response (underlined) in the same column, as determined by interaction chi-square analysis (Steel & Torrie, 1960).

differ in CVI988-vaccinated chickens even though robust differences were noted in bivalent-vaccinated chickens. Thus it does not appear that the use of CVI988-vaccinated chickens will assist discrimination between the vv and vv+ pathotypes. However, CVI988-vacci-

nated chickens might be good models to detect new, but as yet unrecognized, pathotypes.

To test the possibility that more recently isolated MDV strains might have special pathogenicity for CVI988-vaccinated chickens (as has been anticipated,

Table 2. *Challenge of CVI988-vaccinated chickens does not discriminate between vv and vv+ pathotypes[a]*

Isolate	Previously published data				New data	
	Pathotype	Virulence rank	Bivalent (HVT+SB-1)		CVI988	
			% MD	% Protection	% MD	% Protection
679B	vv	39.4	9.1	91	0.0	61
595	vv	61.0	46.7	53	34.0	83
615K	vv	62.5	35.5	63	17.6	80
677	vv	67.8	41.9	58	38.2	57
611	vv	70.5	47.1	50	15.2	83
643P	vv	72.0	44.1	56	26.5	71
Md5	vv	57.9	33.1	67	14.7	85
Mean			36.8A	62A	20.9A	74A
645	vv+	79.0	57.6	42	21.2	76
584A	vv+	82.5	67.6	32	17.6	80
675A	vv+	84.8	69.7	30	21.9	76
652	vv+	86.5	72.7	27	30.3	66
671A	vv+	86.8	73.5	26	8.8	90
660A	vv+	88.7	64.7	35	23.5	74
648A	vv+	91.0	81.8	18	21.2	74
Mean			69.7B	30B	20.7A	77A

[a]Seven MDV strains of the vv pathotype and seven MDV strains of the vv+ pathotype were evaluated. Previous data on these strains obtained with bivalent (HVT+SB-1) vaccinated 15×7 ab+ chickens are listed (Witter, 1997). New data were obtained for each virus by challenge of line 15×7 ab+ chickens at 5 days post vaccination with 2000 PFU of CVI988 (see Table 1). The CVI988 strain was originally obtained from Rhone Merieux through Select Laboratories (Witter *et al.*, 1995). The % MD through 8 weeks post challenge was calculated as in Table 1. The % protection is calculated as described in Witter *et al.* (1995). Groups included 17 chickens. Two replicate trials were conducted and the data were pooled. Means in same column identified by different uppercase letters differ ($P < 0.05$).

especially in locations where CVI988 has been used for many years, and which was supported by our preliminary data; Witter, 2001), three vv+ strains isolated between 1990 and 1995 were contrasted with three vv+ strains isolated between 1997 and 1999 at ADOL. In contrast with our preliminary data, the data presented in Table 3 show no differences between old and new isolates in their relative virulence for CVI988-vaccinated chickens. Of course, this does not rule out the possibility that such viruses exist or may emerge in the future. Continued surveillance is warranted.

We conclude that the inclusion of additional groups of CVI988-vaccinated chickens in the standard ADOL design is not warranted at this time. Furthermore, none of the recent isolates tested appear to exhibit a preferential pathogenicity for CVI988-vaccinated chickens. Similarly, Buscaglia reported that CVI988-vaccinated chickens were highly resistant to challenge with the NULP-1 isolate and yielded no data that would alter pathotyping results obtained with other classes of vaccinated chickens (Buscaglia et al., 2004).

Alternative chicken strains. Two trials were conducted to test whether prototype vv and vv+ isolates could be differentiated by biological responses induced in several different chicken strains. The first trial compared the responses of four chicken strains (7 ×6 ab −, N ab+, TK ab+, and 15 ×7 ab −) to challenge at 21 days with

strains Md5 (vv pathotype) and 648A (vv+ pathotype). Five replicate experiments were performed: three with non-vaccinated chickens, one with HVT-vaccinated chickens and one with HVT+SB-1-vaccinated chickens. Mortality and total MD lesion responses were calculated through 8 weeks post challenge. Vaccination was at hatch (2000 PFU/chick) and challenge was at 21 days of age (500 PFU/chicken). The late challenge was designed to lessen the effects of maternal antibodies, which were present in some chicken lines at hatch but not other lines. The data (Table 4) show that TK ab+ chickens discriminated between vv and vv+ isolates when evaluated by total MD tumour response, regardless of the vaccination status in line TK ab+. However, other lines showed only limited differences. Similar results were reported by Schat and coworkers who differentiated GA5 (v pathotype) and RB1B (vv pathotype) viruses in non-vaccinated, genetically resistant N2 chickens but not in three other more susceptible strains (Schat et al., 1981).

The second experiment was to determine whether commercial SPF chickens could be substituted for 15 × 7 ab+ chickens in the standard ADOL assay. Two commercial strains, SPAFAS ab − and Hy-Vac line TK ab −, were obtained. Six MDV strains representing all three pathotypes were used as challenge viruses. The protocol followed that of the classical ADOL assay, except that only a single non-replicated experiment was

Table 3. *Pathogenicity of selected vv+ isolates in chickens vaccinated with bivalent or CVI988 vaccines[a]*

Year isolated	Isolate	Vaccine	n	MD+	% MD mortality	% total MD	% protection	C/B ratio
1999	686	CVI988	33	10	18.2	30.3	69	>10
		HVT+SB-1	34	31	17.6	91.2	6	
		None	33	33	97	100		
1999	690	CVI988	34	4	2.9	11.8	88	1.1
		HVT+SB-1	34	8	0	23.5	76	
		None	34	34	23.5	100		
1997	677	CVI988	31	4	6.5	12.9	87	1.6
		HVT+SB-1	34	15	0	44.1	54	
		None	32	31	56.3	96.9		
1995	648A	CVI988	34	7	17.8	20.6	79	2.5
		HVT+SB-1	32	21	3.1	65.6	32	
		None	33	33	87.9	100		
1994	645	CVI988	34	4	0	11.8	88	3.1
		HVT+SB-1	33	23	0	69.7	28	
		None	34	34	0	100		
1990	584A	CVI988	34	7	2.9	20.6	79	2.0
		HVT+SB-1	34	20	9.1	58.8	39	
		None	34	34	70.6	100		
	None		10	0	0	0		
Summary								
1997 to 1999		CVI988	98	18	9.2	18.4	81	1.8
		FC126/2+SB-1	102	54	5.9	52.9	45	
		None	99	98	58.6	99		
1990 to 1995		CVI988	102	18	6.9	17.8	82	2.5
		FC126/2+SB-1	99	64	4	64.6	33	
		None	101	101	52.5	100		

[a]To determine whether recent MDV isolates of the vv+ pathotype have increased virulence for CVI988-vaccinated chickens, three vv+ isolates (1997 to 1999) and three vv+ isolates (1990 to 1995) were used to challenge 15 ×7 ab+ chickens that were vaccinated at hatch with the respective vaccine (none, CVI988, and HVT+ SB-1). Vaccine and challenge doses were 2000 and 500 PFU, respectively. MD lesion responses were measured through 8 weeks post challenge. Each treatment group was 17 chickens; two replicate trials were conducted and the data were pooled. Data are presented for each virus, and also as pooled data for each group of viruses. The C/B ratio, calculated as the % protection by CVI988 divided by the % protection by HVT+SB-1 vaccines, is presented.

Table 4. *Discrimination between vv and vv+ isolates by challenge of different lines of chickens at 3 weeks of age[a]*

Trial	Vaccine	Chicken[b]	Number of chicks		% total MD response	
			648A (vv+)	Md5 (vv)	648A (vv+)	Md5 (vv)
1 to 3	None	7 ×6 ab −	84	86	93	87
		N ab+	79	85	94	84
		TK ab+	73	73	100	53
		15 ×7 ab −	86	85	94	99
4	HVT	7 ×6 ab −	32	32	22	3
		N ab+	23	26	22	0
		TK ab+	24	23	67	9
		15 ×7 ab −	32	32	91	72
5	HVT+SB-1	7 ×6 ab −	28	30	4	0
		N ab+	24	18	4	0
		TK ab+	30	28	63	4
		15 ×7 ab −	29	32	41	6

[a]Four types of chickens were challenged at 3 weeks of age with 500 PFU vv (Md5) and vv+ (648A) pathotypes of MDV. Chickens of lines 7 ×6, N and 15 ×7 were from parents maintained at ADOL; line TK was provided by Hy-Line International. Although lines N and TK were derived from parents vaccinated for MD, maternal antibodies were assumed to be largely depleted by the time of challenge. Five experiments were conducted: three with non-vaccinated chickens, one with HVT-vaccinated chickens and one with HVT+SB-1-vaccinated chickens. Each experiment was conducted as two replicates. Data were pooled for each vaccine type. The % MD responses were measured at 8 weeks post challenge and include mortality from early mortality syndrome, transient paralysis and MD lymphomas, as well as birds positive for MD lymphomas at the conclusion of the trial. The responses that best discriminate between vv and vv+ strains are shaded.

performed with each of the chicken lines. Data obtained were compared with those obtained in previous trials using the classical ADOL assay in 15 ×7 ab+ chickens.

Data from SPAFAS chickens are presented in Table 5. Although the magnitude of MD responses was lower in general compared with 15 ×7 ab+ chickens, the pattern of responses in SPAFAS chicken followed closely that obtained by the classical ADOL assay. Correlations with prior data for five response parameters in SPAFAS chickens all exceeded 0.8.

Data from Hy-Vac line TK chickens are presented in Table 6. Although the susceptibility of this line appeared slightly less than that of SPAFAS and con-

siderably less than that of 15 ×7 ab+ chickens, the pattern of responses again appeared comparable with those obtained by the classical ADOL assay. Correlations with prior data for the five response categories was generally good (except for virulence rank), but were not as good as those obtained with SPAFAS chickens.

These results suggest that MDV pathotypes may be successfully differentiated in chickens of alternative genotypes. Buscaglia was able to type viruses successfully using P2a and N2a chickens (Buscaglia *et al.*, 2004). However, Burgess correctly points out that host genetic background can be important when pathotyping MD viral isolates (Burgess *et al.*, 2001)

Table 5. *Pathotyping data in SPAFAS ab− chickens using standard ADOL assay protocol: correlations and best fit pathotype[a]*

Virus	15 ×7[b]		% MD			% mortality		
	Pathotype	Virulence rank	HVT vaccinated	Bivalent vaccinated	Not vaccinated	Not vaccinated	Virulence rank	Best fit
JM/102W	v	17	17.6	5.9	76.5	29.4	15	
Md5	vv	56	23.5	18.8	88.2	70.6	24	
648A	vv+	84	35.3	41.2	100.0	100.0	38	
571A	v	19	5.9	7.7	81.3	18.8	8	v[c]
549A	vv	54	47.1	23.5	94.1	94.1	35	vv+[d]
660A	vv+	89	64.7	47.1	100.0	100.0	56	vv+[e]
Correlation coefficient[f]			0.82	0.98	0.90	0.94	0.92	

[a]Chickens obtained from SPAFAS, Inc. were utilized in the ADOL pathotyping assay (see Table 1) with the following modifications. The chickens were from non-vaccinated parents (ab−). Three prototype control strains were used: JM/102W, Md5 and 648A representing the v, vv and vv+ pathotypes, respectively. Only one replicate trial was conducted and each group contained about 17 chickens which were vaccinated at hatch, challenged at 6 days post vaccination and terminated 8 weeks post challenge. Bivalent vaccine, HVT+SB-1.
[b]Data from earlier trials with replicate lots of 17 chickens per treatment.
[c]Good fit with JM/102W by all criteria.
[d]Fits between Md5 and 648A by most criteria, assigned vv+ on basis of HVT and virulence rank.
[e]Good fit with 648A by all criteria, exceeds 648A by some criteria.
[f]Compared against virulence rank value determined in 15 ×7 chickens by an excel program.

Table 6. *Pathotyping data in Hy-Vac line TK ab– chickens using standard ADOL assay protocol: correlations and best fit pathotype*[a]

| Virus | 15 ×7[b] | | % MD | | | % mortality | | |
	Pathotype	Virulence rank	HVT vaccinated	Bivalent vaccinated	Not vaccinated	Not vaccinated	Virulence rank	Best fit
JM/102W	v	17	5.9	0.0	41.2	11.8	7	
Md5	vv	56	11.8	11.8	88.2	41.2	13	
648A	vv +	84	50.0	18.8	100.0	94.1	34	
571A	v	19	29.4	26.7	47.1	23.5	68	v[c]
549A	vv	54	11.8	11.8	100.0	100.0	13	vv[d]
660A	vv +	89	35.3	31.3	100.0	94.1	33	vv +[e]
Correlation coefficient[f]			0.62	0.48	0.91	0.86	−0.09	

[a] Chickens of Hy-Vac line TK ab − were utilized in a pathotyping assay as described for Table 5. Data are based on single lots of 17 chickens vaccinated at hatch, challenged at 6 days post vaccination and terminated 8 weeks post challenge. Bivalent vaccine, HVT + SB-1.

[b] Data from earlier trials with replicate lots of 17 chickens per treatment.

[c] Good fit with JM/102W in not vac chickens, but exceeds JM/102W by other criteria.

[d] Good fit with Md5 by most criteria, but % mortality most closely resembles that of 648A.

[e] Good fit with 648A by most criteria.

[f] Compared against virulence rank value determined in 15 ×7 chickens by an excel program.

The "Best Fit" Concept

Given that several different lines of chickens appear useful in differentiation of pathotypes, a need exists for standardization of responses to account for susceptibility differences between chicken lines. An obvious approach is to compare responses with those of prototype challenge viruses representing the three pathotypes. The appropriate pathotype would be assigned based on the prototype virus response that best matches that of the test virus. This process of "best fit" can be done statistically, but should also be possible by visual inspection of the data. This concept is illustrated with data from the trial already presented (Tables 5 and 6).

In each set of data, one should first study the responses of the three prototype control strains, in this case JM/102W (v), Md5 (vv) and 648A (vv +). Strain 571A appears by visual comparison to match best with JM/102W in both datasets, especially when one considers data obtained in non-vaccinated chickens; this strain can thus be designated as pathotype v. Strain 549A is a bit more problematic. In SPAFAS chickens, this strain fits between Md5 and 648A by most criteria, but was designated as vv + based on responses in HVT-vaccinated chickens and by the virulence rank parameter. In Hy-Vac line TK chickens, this strain fits best with Md5 by most criteria and, even though mortality in non-vaccinated chickens was closer to that of 648A, it was designated as vv pathotype. Strain 660A data was an excellent fit with that of 648A in both types of chickens and was designated as vv + pathotype. This illustrates the application of the "best fit" method. Strains 571A and 660A were properly classified in both lines of chickens. Strain 549A was properly classified in Hy-Vac line TK chickens, but not in SPAFAS chickens. This strain is probably intermediate in pathogenicity between vv and vv + prototype strains and, thus, may be more difficult to classify.

This suggests that a visual best fit method using commercially available SPF chickens could yield similar pathotype rankings as the standard ADOL method. Additional modifications to reduce the numbers of chicken required are illustrated (Table 7). Instead of conducting a complete, balanced experiment in two replicates, one could first screen isolates in non-vaccinated chickens (Qualitative test A) to determine pathogenicity relative to a prototype v pathotype strain. Viruses that closely resemble the v prototype could be classified without further study. This assay would also identify and properly classify avirulent strains. Only those isolates that are clearly more virulent than the v prototype would be tested in a second screen in two types of vaccinated chickens (Quantitative test B). The total number of chickens required could be less than with the balanced experiment. Furthermore, one could elect to conduct only a single replicate, although we have learned that the precision of the standard ADOL test is improved through replication.

Alternative Criteria

The best fit method using commercial SPF or other chickens is also adaptable, in principle, for use with alternative response criteria. The principle of comparison of responses with those induced by prototype virus strains is independent of the specific criterion used. This concept adds important flexibility to the assay, although one should be cautious when substituting alternative response criteria where the correlation with pathotype is not well documented. Several such alternative criteria are now discussed.

Immunodepression. Calnek *et al.* (1998) reported a strong relationship between pathotype and persistence of early cytolytic infection, viraemia titres, and relative lymphoid organ weights. It appeared that, compared with low virulence strains, viruses of greater virulence caused more persistent and higher titre infections, and induced greater atrophy of the bursa of Fabricius and thymus. These effects were apparent as early as 8 to 10 days post inoculation in both P2a and N2a chickens. The authors suggested use of organ weight as a suitable criterion to determine the pathotype.

Collaborative work was undertaken to extend these findings. In the first set of experiments, a battery of 19 selected ADOL virus strains representing all three pathotypes were provided for challenge in P2a and 15 ×7 ab − chickens at Cornell University (Table 8).

Table 7.   *Two-step experimental design for pathotyping by best fit method*

Test	Lot	Chicks	Chicken strain	Vaccine 2000 PFU	Virus challenge			Test duration
					Strain	PFU	Age	
A[a]	1	10	SPF, ab −	None	Virus 1[c]	500	1 day	56 pcd
	2	10			JM/102W	500	1 day	
	3	10			Md5	500	1 day	
	4	10			None	500	1 day	
B[b]	1	14	SPF, ab −	HVT	Virus 1[c]	500	5 pvd	56 pcd
	2	14			JM/102W	500	5 pvd	
	3	14			Md5	500	5 pvd	
	4	14			648A	500	5 pvd	
	5	14			None	500	5 pvd	
	6	14		HVT+S2	Virus 1[c]	500	5 pvd	
	7	14			JM/102W	500	5 pvd	
	8	14			Md5	500	5 pvd	
	9	14			648A	500	5 pvd	
	10	14			None	500	5 pvd	

[a]Test method A: compare mortality and tumour response to controls. Viruses that resemble JM/102W more closely than Md5 are designated as pathotype v. Viruses that induce no response are classified as nonpathogenic. Other viruses are tested further (Test B). pvd, post vaccination day; pcd, post challenge day.

[b]Test method B (viruses with greater virulence than JM): compare mortality and tumour resonse to controls. Consider data from both vaccinated lots. Strains that best resemble JM/102W are classified as pathotype v. Strains that best resemble Md5 are classified as pathotype vv. Strains that best resemble 648A are classified as pathotype vv+. For strains with measurable virulence but less than that of JM, revise design to include JM/102W and CU-2 as prototype controls (omitting Md5 and 648A).

[c]Additional viruses are tested by inserting additional treatment groups.

Although there was a slight but consistent tendency for v pathotype strains to induce less organ atrophy than other pathotypes at 8 days post inoculation, significant differences in mean bursa and thymus weights were not observed between viruses of the different pathotype groups in either type of chicken. Relative organ weight

Table 8.   *Lymphoid organ weights in chickens inoculated with serotype 1 MDVs[a]*

Pathotype	Strain	15 ×7 (ab −)			P2a (ab −)		
		Number of trials	Bursa weight	Thymus weight	Number of trials	Bursa weight	Thymus weight
v	JM/102W	3	74.0	62.7	5	71.8	47.6
	571A	2	67.0	62.5	3	74.0	60.3
	596A	2	63.0	59.0	3	76.3	59.7
	617A	2	56.5	61.5	1	108.0	80.0
vv	Md5	3	66.0	61.7	5	65.0	53.4
	RB1B	0			1	62.0	43.0
	549A	2	57.0	58.5	3	68.3	53.7
	587A	2	60.5	59.5	3	67.3	70.3
	595	0			2	52.5	35.0
	643P	0			2	66.0	61.5
	653A	2	47.5	41.5	1	75.0	51.0
vv+	584A	0			2	66.0	51.5
	610A	2	57.5	63.5	1	88.0	66.0
	645	2	49.0	44.5	3	74.3	50.3
	648A	0			2	64.5	46.0
	648B	2	55.5	57.5	1	70.0	55.0
	651	0			2	68.0	59.5
	660A	0			2	57.0	46.0
	776	0			2	67.0	65.5
Group means							
v	4	4	65.1A	61.4A	4	82.5A	61.9A
vv	7	4	57.8A	55.3A	7	65.2A	52.6A
vv+	8	3	54.0A	55.2A	8	69.4A	55.0A

[a]Trials were conducted at Cornell University using previously described techniques (Calnek *et al.*, 1998). Chickens were inoculated at 14 days of age with about 500 PFU of virus. Organs were harvested 8 days post inoculation, weighed and converted to relative weights. Data are expressed as the percent of control relative weights. Six trials were conducted; three with P2a ab − chickens, one with 15 ×7 ab − chickens, and two with both chicken types. Not all viruses were tested in each trial (see table for number). The number of chickens per treatment group varied from three to eight (trials 1 and 2) and from nine to 10 (trials 3 to 6). The RB1B stock was from Cornell. All other stocks were supplied by ADOL. Means followed by different uppercase letters within the same column re different (*P* <0.05) by Bonferroni *t* test (Games, 1977).

values for vv and vv+ pathotype strains were virtually identical.

A second collaborative experiment was conducted at ADOL to evaluate the variability of MDV isolates for immunodepressive properties, to determine the relation between immunodepression and pathotype, and to determine the effect of HVT vaccination on immunodepression. Eight selected MDV strains representing all three pathotypes were used to challenge non-vaccinated and HVT-vaccinated chickens of lines 15 ×7 and TK.

Data presented in Table 9 show that, in non-vaccinated chickens, viruses of all pathotypes induced marked depression of relative lymphoid organ weights at 8 days post challenge; mean weights of bursa and thymus in both strains of chickens varied from 48% to 60% of control values (see non-vaccinated mean). However, correlations with virulence rank data obtained in prior assays were poor. HVT vaccination appeared to completely abrogate the atrophic effects of MDV challenge on the bursa and greatly reduced the atrophic effects on the thymus. Correlations with virulence rank data were poor. These data do not support the thesis that organ weights will discriminate between MDV pathotypes on a consistent basis.

Reproducibility of organ weight data can be examined for four virus strains (571A, 596A, 610A and 648B) tested in 15 ×7 ab− chickens in the two sets of trials (Tables 8 and 9). Agreement was not particularly good as one of four bursal weights and three of four thymus weights were markedly lower in the second trial (Table 9) compared with the first trial (Table 8). Another problem was the identification of what appeared to be improperly inoculated chickens in the second trial. Sporadically, in non-vaccinated groups challenged with MDV strains, a chicken would have lymphoid organ weights identical to that of the control whereas all other chickens would have markedly depressed organ weights. As this phenomenon seemed to be correlated with the inoculation technique, non-responder chickens were excluded from the data summary (Table 9). Problems such as these would need to be resolved before lymphoid organ weight parameters could be used with confidence.

Since the immunodepressive potential of MDV strains as estimated from relative organ weights was limited or absent in HVT-vaccinated chickens, immunodepressive properties may not greatly influence pathotyping assays if the assays are conducted in chickens immunized with HVT or other MD vaccines. The possibility that pathotyping data obtained from non-vaccinated chickens might not correlate well with that obtained from vaccinated chickens, because immunodepressive factors may be more important in the former compared with the latter, is an intriguing issue but has not been yet investigated.

In summary, it appears that a relationship between relative bursa and thymus weights and virulence as previously reported (Calnek et al., 1998) is not consistently expressed with all virus strains. Thus, the value of this criterion for pathotyping is uncertain at best, and deserves additional validation prior to use.

Neuropathotyping. Gimeno et al. (2002) reported on a statistical approach to the analysis of clinical neurological responses to 29 different MDV strains of known pathotype. Three clusters (groups) were derived and were designated A, B and C according to ascending grades of neurological response. There was a clear relation between neuropathotype and pathotype. For example, neuropathotype A contained the JM/102W strain (prototype v pathotype), neuropathotype B contained the Md5 strain (prototype vv pathotype) and neuropathotype C contained the 648A strain (prototype vv+ pathotype). However, neuropathotype C included most of the vv pathotype strains in addition to all vv+ pathotype strains. Table 10 illustrates the relation between pathotype and neuropathotype. There is a very good fit between pathotype v and neuropathotype A, and between pathotype vv+ and neuropathotype C. Pathotype vv, however, includes all three neuropathotypes, although most are concentrated in either neuropathotype B or C. The authors suggest neuropathotyping as an adjunct rather than a replacement for traditional pathotyping (Gimeno et al., 2002).

Neuropathotyping was also conducted successfully in SPAFAS and Hy-Vac line SC chickens, indicating again that pathotypic differences can be measured in chickens of various, but not all, genotypes (Gimeno et al., 2002). Even though the statistical method for assignment of neuropathotype groups did not fit the existing pathotype classification, the correlation between neurological clinical signs and virulence was strong. Neurological responses induced by each of the three prototype viruses were well differentiated from each other. Thus, neurological responses should be useful for pathotyping when compared with prototype strains by the best fit method.

Virus replication and virus load. Some workers have noted that isolates of higher virulence, especially vv+ strains, tend to grow faster and produce higher virus titres in inoculated chickens compared with isolates of lower virulence (Rosenberger, 1995). Calnek and coworkers noted such a relationship between pathotype and the number of virus plaques induced in cell cultures inoculated with splenocytes obtained 4 to 8 days post inoculation (Table 11). Others have observed similar trends with samples collected at various time periods. However, plaque assays in cell culture to detect cell-associated infectivity yield highly variable data, making it difficult to detect small differences between groups of samples. Quantitative PCR assays to detect viral DNA offer a more sensitive approach to the determination of virus load in various tissues (Bumstead et al., 1997; Burgess & Davison, 1999; Reddy et al., 2000). Yunis et al. (2004) have used a real-time reverse transcriptase-PCR assay to detect viral transcripts against gB and ICP4/LAT produced during 1 to 10 days post infection (d.p.i.). The data differentiated the prelytic period (d.p.i. 1 to 3) when transcripts were barely detectable, the lytic phase (d.p.i. 4 to 5) when both transcripts were clearly detectable in JM-16, and the latent phase (d.p.i. 6 to 10) when transcript levels were low in JM-16-infected chickens. Comparisons between the JM-16 strain (v pathotype) and RK-1 (vv+ pathotype) clearly illustrate the greater in vivo replication potential for vv+ isolates during the lytic and latent phases of infection (Table 11). Although the relationship between virus replication or virus load and pathotype has not been rigorously tested, it appears that these parameters are correlated and that values for prototype strains may differ. Thus, the use of virus load as a response criterion for comparison with prototype strains in a best fit assay could be considered. Timing, however, could be critical. Some reports show

Table 9.   *Relationship between virulence and lymphoid organ weight depression[a]*

Virus	Pathotype	Virulence rank	Bursa weights (% of control)				Thymus weights (% of control)				Non-vaccinated mean
			Non-vaccinated		HVT vaccinated		Non-vaccinated		HVT vaccinated		
			15 ×7	TK	15 ×7	TK	15 ×7	TK	15 ×7	TK	
571A	v	19.5	67.6	80.7	95.2	95.3	46.6	39.2	73.6	83.0	58.5
596A	v	28.5	39.5	83.5	93.1	99.2	34.9	50.2	75.5	83.6	52.0
653A	vv	42.5	49.5	60.2	90.9	106.1	26.8	57.8	69.2	118.2	48.6
595	vv	61.0	50.2	76.9	101.0	120.1	44.1	36.6	78.2	86.9	52.0
610A	vv+	79.5	56.1	52.6	85.5	113.7	30.5	76.1	72.3	107.8	53.8
676	vv+	85.0	44.1	75.9	99.5	110.6	39.7	69.9	85.2	67.4	57.4
648B	vv+	87.0	57.7	63.9	81.4	113.6	54.6	64.3	75.6	65.9	60.1
660A	vv+	89.0	50.3	59.6	95.2	122.0	34.0	57.5	76.4	107.4	50.4
Correlation coefficient[b]			−0.12	−0.59	−0.21	0.84	0.09	0.66	0.43	−0.10	0.14

[a]Eight MDV strains, representing all three pathotypes and selected to include viruses that appeared to differ for immunodepressive properties, were used. Groups of chickens of line 15 ×7 ab − and Hy-Vac line TK ab − that differed in MD susceptibility were either vaccinated at hatch with 2000 PFU of HVT or were left unvaccinated. All chickens were challenged at 7 days post vaccination with 500 PFU of the respective MDV strain. Non-challenged control lots for each vaccine and chicken type were also used. Chickens were killed 8 days post challenge. Body weight, relative bursa weight and relative thymus weight were determined for each chicken as described (Witter *et al.*, 1997). The trials were conducted in four replicates; two replicates with 15 ×7 ab − chickens and two replicates with TK ab − chickens. Each treatment group contained about eight chickens. Weights are expressed as a per cent of control weight. The non-vaccinated mean is the mean of all non-vaccinated values, including both organs and both chicken strains. A small proportion of non-vaccinated, MDV-challenged chickens had normal organ weights and differed from other treated chickens in the same group. These were considered to be non-responders due to errors in inoculation and the data were excluded.

[b]Compared with virulence rank values (determined from earlier trials).

Table 10. *Relation between pathotype and neuropathotype[a]*

Pathotype	Number of strains	Number of strains classed as neuropathotype[b]		
		A	B	C
v	6	5	1	0
vv	13	1	4	8
vv+	10	0	0	10

[a]Data from Gimeno *et al.* (2002).

[b]Neuropathotype classification: A, variable classical transient paralysis (TP), low TP with mortality before 15 days (acute TP), low persistent neurological disease (neurological signs starting at 19 days or later) (PND); B, hi TP, low acute TP, high PND; C, high TP, high acute TP, high PND.

differences in viral titres between pathotypes are more evident at later ages, probably reflecting the generation of tumour cells, than during the initial cytolytic phase (Schat *et al.*, 1982).

controls would be indicated (see Table 7). Alternatively, inoculation of 8-day embryos may provide a more susceptible host system for evaluation of low virulence strains (Calnek *et al.*, 1977).

Pathotyping Mild Strains

A pathotypic classification of mildly virulent (m) was proposed (Witter, 1997) to include serotype 1 strains such as CU2 (Smith & Calnek, 1974), B14 (Biggs & Payne, 1963) or other classical isolates (Biggs & Payne, 1967; Biggs & Milne, 1972). Such strains are less virulent than viruses of the v pathotype such as the JM, GA and HPRS-16 strains. The ADOL method is not appropriate for such strains without modification; namely, the inclusion of a control virus representative of the m pathotype. At present, low virulence viruses are rarely, if ever, isolated from field cases. It is important to differentiate low virulence strains from apathogenic strains of serotype 2. However, if a strain appears much less virulent than JM and contains serotype 1 antigens, a follow-up test in non-vaccinated, MD-susceptible chickens using prototype v and m strains as

Prototype Strains

The best fit method requires that data be compared with those of prototype strains. However, the use of prototype strains can also provide a powerful and simple method to standardize results in different laboratories, providing that the same set of strains is utilized. We suggest three prototype strains for consideration: JM/102W (v pathotype), Md5 (vv pathotype) and 648A (vv+ pathotype). The JM/102W and Md5 strains are currently on deposit with the American Type Culture Collection (ATCC), Rockville, Maryland, USA. The 648A strain has been accepted for deposit by the ATCC. These strains may also be obtained from ADOL. Characteristics of these strains are described in Table 12. It is important, of course, that these strains be kept at very low cell culture passage to avoid modification of virulence through attenuation. Other strains could also be used as controls,

Table 11. *Relationship between pathotype and virus load*

Trial	Virus strain	Pathotype	Chicken strain	Days post challenge	PFU/10^6 leukocytes	Real-time reverse transcriptase PCRz	
						ICP4/LAT	gB
A[a]	JM-16	v	P2a	4 to 8	14.4±2.7		
	RB1B	vv	P2a	4 to 8	41.2±5.5		
	RK1	vv+	P2a	4 to 8	30.3±7.3		
B[b]	JM-16	v	N2a	1 to 3		0.017	0.045
	RK-1	vv+	N2a	1 to 3		0.008	0.012
	JM-16	v	N2a	4 to 5		0.483	0.577
	RK-1	vv+	N2a	4 to 5		0.736*	0.752
	JM-16	v	N2a	6 to 10		0.039	0.196
	RK-1	vv+	N2a	6 to 10		0.316*	0.509*
C[c]	R2/13	Virulent	15 ×7 ab −	14	137		
	R2/22	Virulent	15 ×7 ab −	14	127		
	R2/23	Avirulent	15 ×7 ab −	14	19		
	R2/29	Avirulent	15 ×7 ab −	14	31		

[a]Data from Calnek *et al.* (1998). Data are pooled from 30 birds/group over 4 to 8 days post inoculation. Leukocytes were obtained from spleens.

[b]Data from Schat and coworkers (Yunis *et al.*, 2004). RNA was harvested from spleens between 1 and 10 days post infection (d.p.i.) and the levels of ICP4/LAT and gB transcripts were measured by real-time reverse transcriptse-PCR. Data are the mean number of ICP4/LAT and gB transcripts during the prelytic (1 to 3 d.p.i.), lytic (4 to 5 d.p.i.) and latent (6 to 10 d.p.i.) period. The values were normalized for cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript copies. * Significantly different at $P < 0.05$ between JM-16 and RK-1 for the same period post infection.

[c]Data from Witter (1991). Data are from four clones of Md11/75C/R2 that vary in virulence. Leukocytes were obtained from buffy coat preparations.

Table 12.   *Suggested serotype 1 prototype strains for best fit pathotyping*

Strain	Pathotype	Virulence rank	Clone	Passage	ATCC number	Neuropathotype	Reference
JM/102W	v	17.8	Yes	11 to 13 DEF	VR-585	A	Sevoian *et al.* (1962), Stephens *et al.* (1976)
Md5	vv	55.7	No	5 to 7 DEF	VR-987	B	Witter *et al.* (1980)
648A	vv+	80.9	No	6 to 8 DEF	VR-1576	C	Witter (1997)

providing they are first rigorously compared with the aforementioned prototype strains and established to be representative of the respective pathotypic group. However, there is a caveat to this approach. Since virulence of MDVs represents a continuum, no two viruses will probably be identical. Thus an alternative control virus will probably represent a different spot in this continuum and inevitably bias the result. For this reason, use of alternative strains should be reserved for situations where standard prototypes cannot be obtained.

In principle, prototype strains always should be cloned. However, the fact that Md5 and 648A are not cloned should not affect their suitability as controls for pathotyping assays. If these viruses are cloned in the future, it would be important to compare the pathogenicity of the clones with the parent strains.

Conclusions and Recommendations

A modification of the standard ADOL assay based on the use of commercial SPF (or other) chickens should be adaptable to many laboratories. Furthermore, the procedure can be enhanced when responses are compared with those of prototype control strains by a best fit method. Measurement of 8-week tumour responses should ensure that the data will be relevant to published pathotypes. Widespread use of this method should provide greater standardization of pathotype data generated by different laboratories. This approach has not yet been validated. However, this method is currently being evaluated in Russia and the initial results appear promising (E. Dudnikova, personal communication, 2004).

Although the ability to utilize commercial SPF chickens removes an important obstacle to international use of a standard pathotyping assay, other obstacles may be encountered. Some countries, such as Australia and the United States, have regulations that severely restrict importation of viruses. If prototype strains cannot be imported, the modified assay described here cannot be conducted. One solution would be for tests to be conducted in a third country that has access to the ADOL prototype strains and a liberal policy for receiving field isolates from foreign countries. Once local MD viral isolates have been stringently compared with the prototype strains, then one or more local isolates could be used to control pathotyping tests in the country of origin.

There is a need for more work on alternative criteria for pathotyping, especially viral replication and viral load (*in vivo*). Although organ weights and neurological signs appear to have limited applicability, parameters of viral replication have not yet been sufficiently investigated as a differentiation tool. These criteria are attractive because data can be obtained in a much shorter time using fewer chickens that do not develop tumours, factors that would facilitate approval of pro-

tocols by Institutional Animal Care and Use Committees.

Finally, it is important to consider whether, in the course of future studies, specific molecular changes in the viral genome will be identified that will offer definitive and rapid means for pathotyping. Thus far, there is no indication that virulence of MDV has a simple genetic basis. Comparisons of virulent and attenuated strains have revealed many differences. Expansions of a 132 base pair repeat have been associated with attenuation (Fukuchi *et al.*, 1985; Silva & Witter, 1985), but this is now known to be unrelated to virulence (Silva *et al.*, 2004). Mutations in the *meq* gene have been associated with variations in virulence (Chang *et al.*, 2002) but there are insufficient data to justify use of such criteria for pathotyping. This issue awaits the dissection of the molecular basis of viral virulence—a critical and complex issue that will remain a high priority in MD research for the foreseeable future.

Validation of alternative criteria should be based on tests with multiple representative strains of each pathotype. The ADOL virus collection, which contains 47 strains of serotype 1 MDV pathotyped by the original ADOL assay, could be useful in such studies.

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Translations of the abstract in French, German and Spanish are available on the *Avian Pathology* website.

Non-English Abstracts

Classification of Marek's disease viruses according to pathotype: philosophy and methodology[1]

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Le concept de pathotype pour la maladie de Marek (MD) date probablement de l'identification d'une forme plus virulente de la maladie à la fin des années 1950 (Benton & Cover, 1957). Les distinctions entre les souches de virus de la MD (MDV) ont été développées plus tardivement avec la description des pathotypes très virulents (vv) au début des années 1980 et des hypervirulents (vv+) dans les années 1990. Les désignations de pathotype reflètent des propriétés biologiques importantes qui correspondent au franchissement de l'immunité vaccinale sur le terrain. Cependant les méthodes de pathotypage, mises en pratique dans différents laboratoires, n'ont pas été les mêmes empêchant la comparaison critique des résultats. Une meilleure harmonisation des procédures de pathotypage est souhaitable.

La méthode du Laboratoire des Maladies Aviaires et d'Oncologie (ADOL) est basée sur l'induction des lésions lymphoprolifératives chez les poulets vaccinés. Cette méthode a été utilisée pour pathotyper plus de 45 souches et est la base de la classification actuelle par pathotype des souches de MDV. Les limites de cette méthode incluent les exigences en ce qui concerne le type de poulets (15x7 ab+), un nombre important d'animaux, et une méthode statistique pour comparer les réponses lésionnelles à celles des souches témoins JM/102W et Md5. Du fait de ces limites, cette méthode n'a pas été utilisée dans d'autres laboratoires, et ne sera probablement pas.

La comparabilité du pathotypage peut être améliorée par la comparaison des souches du terrain à des souches prototype standard, telles la JM/102W, la Md5 et la 648A (American Type Culture Collection) ou à des souches équivalentes. Les données peuvent être générées par différentes procédures *in vivo* qui mesurent l'induction des tumeurs, la maladie neurologique (les lésions néoplastiques et non-néoplastiques), ou seulement les critères non-néoplastiques (tel les poids des organes lymphoïdes ou la réplication virale). Les méthodes basées sur les critères néoplastiques, particulièrement quand ils apparaissent chez des poulets immunisés MD, devraient probablement correspondre plus étroitement à celles de la méthode d'ADOL et être plus en rapport avec l'évolution des virus sur le terrain. A partir des données de plusieurs essais, une modification de la méthode de l'ADOL est proposée. Elle utilise moins de poulets et peut être réalisée avec des variétés commerciales de poulets SPF. Cette méthode modifiée est basée sur de meilleures comparaisons des souches prototypes, et on peut s'attendre à fournir des résultats généralement comparables à ceux de la méthode originale. D'autres critères alternatifs (Cf.supra) sont également évalués aussi bien pour le premier pathotypage que comme compléments à d'autres méthodes de pathotypage. Les avantages et les inconvénients de ces méthodes alternatives sont présentés.

Der Begriff des Pathotyps bei der Marekschen Krankheit (MK) entstand wahrscheinlich im Zusammenhang mit dem Auftreten einer virulenteren Form der Erkrankung in den späten 1950iger Jahren (Benton &Cover, 1957). Die Unterscheidung zwischen verschiedenen MK-Virus (MKV)-Stämmen wurde mit der Beschreibung des vv-Pathotyps in den frühen 1980iger Jahren und des vv+-Pathotyps in den 1990iger Jahren weiter ausgedehnt. Diese Pathotypbezeichnungen reflektieren wichtige biologische Eigenschaften, die mit Impfdurchbrüchen im Feld im Zusammenhang stehen. Die Pathotypisierungsmethoden in verschiedenen Laboratorien waren jedoch nicht einheitlich, was einen tatsächlichen Vergleich der Ergebnisse bislang verhinderte. Aus diesem Grund ist die Vereinheitlichung der Pathotypisierungsverfahren wünschenwert.

Die Methode des ‚Laboratoriums für Vogelkrankheiten und −onkologie' (Avian Disease and Oncology Laboratory (ADOL)) basiert auf der Induktion lymphoproliferativer Läsionen in vakzinierten Hühnern.. Diese Methode wurde für die Pathotypisierung von mehr als 45 Isolaten verwendet und ist die Basis für die

derzeitige Pathotyp-Klassifizierung von MKV-Stämmen. Ihre Anwendung ist eingeschränkt aufgrund des Erfordernis eines bestimmten Hühnertyps (15x7 ab +), einer großen Anzahl von Versuchstieren und einer statistischen Methode, die den Vergleich der auftretenden Läsionen mit denjenigen durch die Kontroll-stämme JM/102W und Md5 ermöglicht. Aufgrund dieser Einschränkungen war und ist diese Methode nicht für die Anwendung in anderen Laboratorien geeignet.

Die Vergleichbarkeit der Pathotypisierung kann durch den Vergleich von Feldisolaten mit Standardproto-typstämmen wie JM/102W, Md5 und 648A (American Type Culture Collection) oder ihrer Äqivalente verbessert werden. Die entsprechenden Daten können durch zwei verschiedene *in vivo*-Verfahren gewonnen werden, die entweder die Tumorinduktion und die neurologische Erkrankung (sowohl neoplastische als auch nicht-neoplastische Veränderungen) oder nur nicht-neoplastische Kriterien (wie Gewicht der lymphatischen Organe oder Virusreplikation) ermitteln. Ergebnisse, die auf der Bestimmung neoplastischer Kriterien basieren, insbesondere wenn sie in MK-immunisierten Hühnern durchgeführt werden, werden wahrschein-lich am ehesten mit den nach der ADOL-Methode erhobenen Daten korrelieren und für die Beurteilung der Evolution des MKV im Feld von größter Bedeutung sein. Basierend auf den Daten von verschiedenen Untersuchungen wird eine Modifizierung der ADOL-Methode, bei der weniger Hühner verwendet werden, die außerdem aus kommerziellen SPF-Stämmen sein können, vorgeschlagen. Die modifizierte Methode basiert auf ‟Best Fit"-Vergleichen mit den Prototypstämmen, d.h. mit welchem Prototyp gibt es die größte Übereinstimmung in den Befunden, und es wird erwartet, dass sie Ergebnisse erbringt, die mit denen der Originalmethode generell vergleichbar sind. Eine Vielzahl von anderen Alternativkriterien (siehe oben) wurden ebenfalls auf ihre Eignung sowohl für die Primärpathotypisierung als auch als Ergänzung zu anderen Pathotypisierungsmethoden beurteilt. Vor- und Nachteile dieser Alternativmethoden werden erläutert.

El concepto de patotipo en la enfermedad de Marek (MD) data probablemente de finales de los 1950s cuando se reconoció una forma más virulenta de enfermedad (Benton y Cover, 1957). Las distinciones entre las diferentes cepas de virus de MD (MDV) fueron aún mayores al describirse el patotipo vv a principios de los ochenta y el vv + en los noventa. La designación de patotipo refleja propiedades biológicas importantes que se correlacionan con la capacidad de romper la inmunidad maternal en el campo. A pesar de ello, los métodos de clasificación de los diferentes patotipos en varios laboratorios no han sido uniformes, lo cual ha impedido una comparación crítica de los resultados.

El método utilizado en el *Avian Disease and Oncology Laboratory* (ADOL) se basa en la inducción de lesiones linfoproliferativas en pollos vacunados. Este método ha sido utilizado para clasificar más de 45 aislados y es la base para la clasificación actual de los patotipos de cepas de MDV. Las limitaciones de este método son varias: necesidad de un tipo específico de pollos (15x7 ab +), uso de un gran número de animales y de un método estadístico para comparar las respuestas lesionales con las de las cepas control JM/102W y Md5. Debido a estas limitaciones no ha sido y no es probablemente usado en otros laboratorios. La comparación en el patotipado puede ser mejorada mediante la comparación de aislados de campo con cepas prototipo como las JM/102W, Md5 y 648A (American Type Culture Collection) o sus equivalentes. Los datos pueden ser generados mediante diferentes procedimientos in vivo que miden la inducción de tumores, enfermedad neurológica (por lesiones neoplásicas o no neoplásicas), o únicamente por criterios no neoplásicos (como el peso de los órganos linfoides o la replicación vírica). Los métodos basados en criterios neoplásicos, especialmente cuando son generados en pollos inmunizados de MD, probablemente se correlacionarán mejor con el método del ADOL y serán más relevantes en cuanto a la evolución de los virus de MD en el campo. En base a los datos de diferentes experimentos, se propone una modificación del método ADOL que utiliza menos animales y puede ser llevado a cabo en pollos SPF comerciales. El método modificado se basa en una comparación con el que mejor clasifica las cepas prototipo, y se espera que de resultados en general comparables con el método original. Otros criterios alternativos (ver abajo) también se evalúan como métodos primarios de patotipificación o como adjuntos a otros métodos de patotipificación. Se presentan las ventajas y desventajas de estos métodos alternativos.