Spring 1991

Serologic Testing of Rodents for Viral Infections: Interpretation of Results

Introduction

Two recent bulletins (*A Laboratory Animal Health Monitoring Program: Rationale and Development* and *Serologic Testing to Monitor Rodents for Viral and Mycoplasmal Infection*) have emphasized that laboratory animals adventitiously infected with pathogenic viruses, bacteria and/ or parasites are not suitable for research. This is because adventitious (i.e., accidental) infections may lead to clinical disease and pathological changes, especially in perinatal and immunodeficient animals. Although infections in post-weaning, immunocompetent animals are often subclinical, they can contaminate biological materials or change biological responses and cloud the interpretation of experimental results. Furthermore, some agents indigenous to laboratory animals are zoonotic. While causing asymptomatic infections of their natural hosts, such agents can cause disease and death in human beings.

Charles River Laboratories has pioneered large-scale production of rodents free from infection by specific pathogenic viruses, bacteria and parasites using cesarean derivation (mice and rats only) and barrier maintenance. To further prevent infections, these "virus antibody-free" (VAF®) rodents are shipped in filtered crates. Investigators can maintain the VAF® status by strict colony management that may include the use of barrier rooms, isolators or microisolation units. However, adventitous infections will occur at both breeder and customer facilities, despite the use of rigorous procedures. It is therefore essential to perform routine comprehensive health monitoring employing a variety of diagnostic methodologies.

Viruses are highly contagious and hence most prevalent, while barrier rooms have successfully excluded most pathogenic bacteria and parasites. Routine monitoring for virus exposure is accomplished by serologically testing rodent serum samples for virus-specific antibodies formed as part of the immune response to infection. Serology is used because antibodies are persistent, and assays for their detection are rapid, sensitive and specific when performed properly. Serology is also commonly used to test mice and rats for antibodies to *Mycoplasma pulmonis* (the cause of murine respiratory and genital mycoplasmosis) and a few other non-viral agents.

Traditional serologic tests, such as complement fixation (CF) and hemagglutination inhibition (HAI), have now been supplanted by non-radioisotopic solid phase immunoassays, notably the enzyme-linked immunosorbent assay (ELISA) and the indirect fluorescent antibody test (IFA). The ELISA and IFA are extremely sensitive, and both performance and processing of results can be automated.

Choosing Assays and Animals to Monitor

Valid and useful results depend first on the correct choice of assays and animals to monitor. These decisions are based on considerations detailed in previous issues (as noted at left), which may be summarized as follows:

Routine screening should be conducted for agents found commonly in laboratory rodents, while screening for rarely found agents can be more widely spaced. At Charles River, we perform "Tracking" profiles of tests for antibodies to common viruses (and *M. pulmonis* in mice and rats) on VAF® areas every 66 weeks. More comprehensive "Assessment" and "AssessmentPlus" profiles, including tests for antibodies to rarely found viruses, are performed annually (Table 1).

Table 1. CRL Rodent Serology Profile

SPECIES	PROFILE	AGENTS INCLUDED
Mouse	Tracking	SEN, PVM, MHV, MVM, GD-7, REO-3, MPUL.

	AssessmentPlus	Tracking Profile plus LCMV, MAD, ECTRO, K, POLY, MTLV, MCMV, HANT, ECUN, EDIM, CARB.
Rat	Tracking	SEN, PVM, RCV/SDA, KRV, H-l, REO-3, MPUL.
	AssessmentPlus	Tracking Profile plus GD-7, LCMV, MAD, HANT, ECUN, CARB.
Guinea Pig	Tracking	SEN, SV-5, PVM, REO-3.
	Assessment	Tracking Profile plus LCMV, ECUN.
Hamster	Tracking	SEN, SV-S, PVM, REO-3, LCMV.
	Assessment	Tracking Profile plus ECUN.

The number of animals tested per screening is based on various factors including the estimated prevalence of antibody positive animals, the degree of certainty desired, and the cost. (It is important to distinguish between prevalence and incidence. Prevalence is the percentage of positive animals at a point in time in a designated area. Incidence is the percentage of new positives over a *period* of time.) At Charles River, the usual screening sample includes eight animals.

As for frequency, animals may be screened immediately after shipment to confirm the vendor's health report. We strongly recommend that rodents be screened 2-4 weeks after arrival, to test for exposure in transit or at the destination facility. Thereafter, screening should be conducted at regular intervals.

Obviously, the animals sampled must be immunocompetent and capable of producing detectable levels of antibodies in response to infection. They should have had sufficient time and exposure to respond. Sentinels should be maintained on soiled bedding, as viruses and mycoplasma do not necessarily spread easily via aerosols. From established colonies, retired breeders are good animals to sample because they have had maximum opportunity for exposure and response to any pathogens in the colony. Finally, antibodies require 2-3 weeks to reach detectable levels. Serologic testing of clinically ill animals is not advisable.

Types of Interpretation

Serologic results can be interpreted in various ways. For example, the strength of the immune response can be quantified by measuring antibody titers or reaction intensities. Detection of virus-specific IgM or a four-fold rise in titer in paired serum samples from an animal indicates that active virus infection has recently occurred. Reciprocal antibody titrations with multiple virus strains by tests that measure type-specific antibodies, e.g., the HAI or the neutralization test *(NT)*, may identify the infecting virus strain or serotype. These interpretations may be of value but are not of paramount importance because VAF® rodents, as the acronym indicates, are free of antibodies to specific viruses. Detection of any amount of viral antibodies in these animals, regardless of titer, points to adventitious infection. By doing routine monitoring, the time of exposure can be closely circumscribed. Determining the serotype of the infecting virus may not be useful, as it often does *not* correlate with pathogenicity.

By far the most important interpretation for rodent serology is determining *whether or not infection has occurred*. Making this interpretation is problematic, as no serologic assay always gives accurate results. False positive and negative results are not uncommon. Figure I compares the ideal situation (in which titer or reaction intensities for virus-exposed and virus-free animals are completely separable) with a typical assay. The latter is characterized by a gray zone in which exposed animals show false negative reactions, and unexposed animals show false positive reactions.

Reasons for False Negative Results

False negatives are those negative serologic results that occur in a group of animals known to have been exposed to the virus under scrutiny (i.e., an "exposed" group). A sensitive assay is one that produces a low percentage of false negative results or, conversely, a high percentage of true positive results in tests performed on a virus-exposed group. Assay sensitivity equals the percentage of true positive results in an exposed group (Figure 2). False negative results may occur because:

1. Antibody levels were below those detectable by a particular assay method (i.e., the assay is insensitive).

2. Animals were tested during or shortly after the active phase of infection, before having sufficient time to develop serum antibodies; or, in the case of the less sensitive conventional assays, they were tested too long after infection.

3. The samples tested came from immunodeficient animals (e.g., athymic nude mice) that mounted a minimal or delayed antibody response.
4. Antibodies were formed to a heterologous serotype that did not cross-react in the serotype- or strain-specific assay employed (e.g., HAI and NT).
5. Based on the low prevalence of antibody-positive animals, an insufficient number was tested.
6. The sentinel animals were not maintained on soiled bedding and hence were inadequatedly exposed to viruses in the colony.
7. Samples were not prepared or stored properly.
8. Assay preparation or performance was deficient.

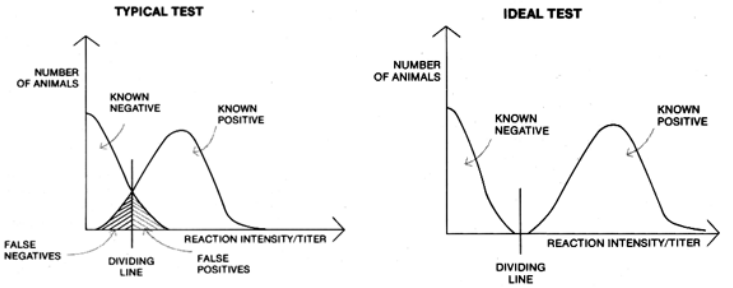


Figure 1. Comparison of typical and ideal serology test.

		VIRUS PRESENT	VIRUS ABSENT
TEST RESULT	"POSITIVE"	TP	FP
	"NEGATIVE"	FN	TN

$$\text{SENSITIVITY} = \frac{TP}{TP + FN} \times 100$$

$$\text{SPECIFICITY} = \frac{TN}{FP + TN} \times 100$$

Figure 2. Definition of assay sensitivity and specificity. (TP/FP = true/false positive; FN/TN = false/true negative.) Adapted from Zweig and Robertson in Chan and Perlsten (eds): **Immunoassay, a Practical Guide** (1987), pp. 97-128, by permission of Academic Press.

Reasons for False Positive Results

False positives are those positive results that occur in a group of animals known *not* to have been exposed to the infectious agent in question (i.e., an "unexposed" group). A specific assay is one that gives a low percentage of false positive results or, conversely, a high percentage of true negative results in tests performed on an unexposed group. Assay specificity is computed as the percentage of true negative results in a group known to be free of a particular virus (Figure 2).

The causes of false positive results are not clearly understood. They occur in the ELISA, IFA, and CF test when antibody in a specimen binds non-specifically to the antigen but not to its tissue control. (In the ELISA and CF, the control is an extract of uninfected tissue or cells of the type in which the virus is propagated. In the IFA, it is uninfected cells.) In the HAI test, false positives occur when the specimen contains non-specific inhibitors of hemagglutination. As with false negatives, false positives may occur because samples are improperly prepared and stored, or the assay is improperly prepared or performed.

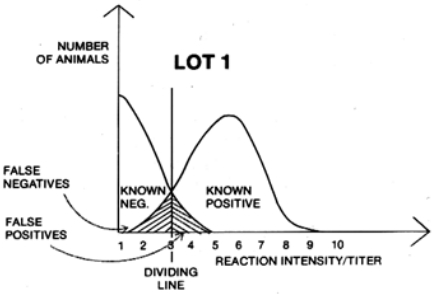


Figure 3(a). Constant dividing line: lot-to-lot variation.

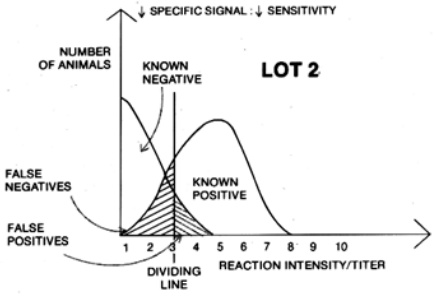


Figure 3(b). Constant dividing line: lot-to-lot variation.

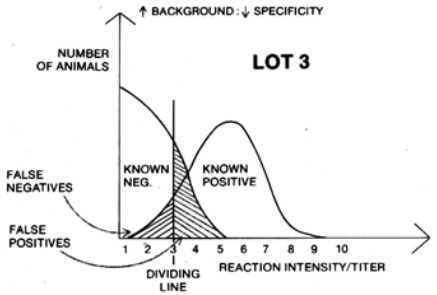


Figure 3(c). Constant dividing line: lot-to-lot variation.

Figures 3a through 3c underscore the point that reaction intensities or titers near the dividing line between positive and negative are those most likely to be inaccurate. Most testing laboratories, including Charles River, use a constant dividing line for quantitative or semi-quantitative tests such as the HAI, CF and ELISA. Depending on line placement, there is a trade-off between sensitivity and specificity. Specificity can be increased at the expense of sensitivity, or *vice versa*. Even with the best controlled tests, some variation will occur in the range of titers given by positive and negative sera due to lot-to-lot variation of reagents, solutions and disposables (Figure 3) or technician and hardware problems. Such inconsistencies stress the need for caution when interpreting results near the dividing line.

The prevalence of antibody-positive animals is another factor to be considered. It affects the predictive value of a positive result, which is the percent of positive results that are true positive. In the example shown in Figure 4, the predictive value of a positive result for an assay with a specificity of 80 percent is evaluated when the prevalence of antibody positives is 10 percent, and again when the prevalence is 1 percent.

When the prevalence is 10 percent, the predictive value of a positive result is 35 percent. In other words, one would expect 65

percent of the positive results to be inaccurate. With a prevalence of 1 percent, only 4.7 percent of the positive results are accurate.

$$PV = \frac{TP}{TP + FP} \times 100$$

DISEASE OR CONDITION

TEST RESULT	PRESENT	ABSENT
"POSITIVE"	9,800	18,000
"NEGATIVE"	200	72,000

TOTAL SUBJECTS = 100,000
PREVALENCE = 10%
SENSITIVITY = 98%
SPECIFICITY = 80%

$$PV(+) = \frac{9800}{9800 + 18{,}000} \times 100 = 35.3\%$$

DISEASE OR CONDITION

TEST RESULT	PRESENT	ABSENT
"POSITIVE"	980	19,800
"NEGATIVE"	20	79,200

TOTAL SUBJECTS = 100,000
PREVALENCE = 1%
SENSITIVITY = 98%
SPECIFICITY = 80%

$$PV(+) = \frac{980}{980 + 19{,}800} \times 100 = 4.7\%$$

Figure 4. Effect of prevalence on the predictive value (PV) of positive results.

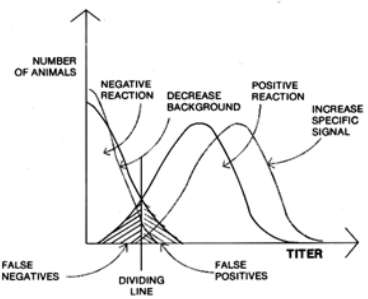
In summary, false positives should be suspected when the reaction intensities or titers are low and/or if the prevalence of positives is low (i.e., less than or equal to 15 percent). They should also be suspected if results cannot be confirmed by the same or alternative assays. Note: False positives can occur due to passively acquired maternal antibodies, which may be detectable for several months after birth.

Ways to Enhance Accuracy of Results

Proper sample preparation and storage are essential for accurate results. The adage, "Garbage in, garbage out," applies to serology as well as to computers. To ensure that good samples are submitted:

Serum should not be bacterially contaminated or badly hemolyzed. It should be removed from clotted blood as soon as clotting is complete and diluted with a sterile, buffered saline solution, pH 7.2 (+/- 0.2).

Figure 5. Assay optimization: increase signal-to-noise ratio.



Serum should be refrigerated or preferably frozen for longterm storage. Samples to be refrigerated for more than a couple of days should be protected by preservative such as thimerosal or sodium azide.

In assay preparation and performance, inaccurate results can be reduced (i.e., sensitivity and specificity can be improved) by increasing the signal-to-noise ratio; that is, by increasing the intensity/titer of reactions given by immune sera while decreasing the background reactions given by non-immune sera (Figure 5). In the CRL ELISA, this is achieved by increasing antigen purity so that more specific antibody can bind to the solid-phase (Figure 6) and by blocking non-specific binding of immunoglobulin with a special diluent (Figure 7).

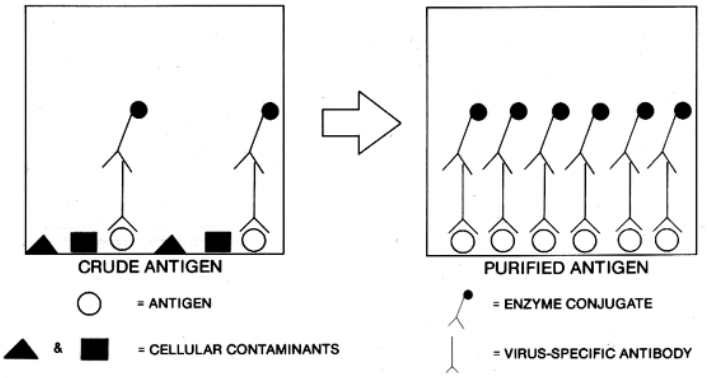


Figure 6. Increasing signal-to-noise ratio: antigen purification.

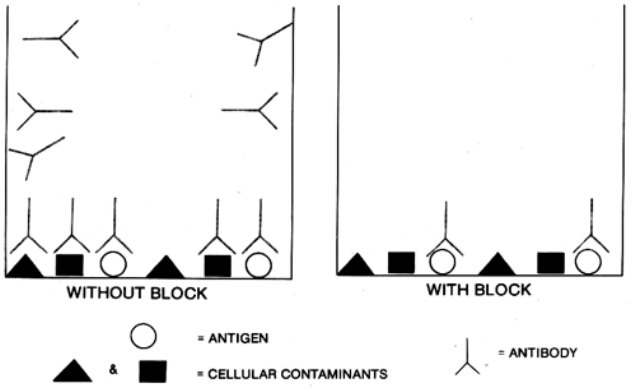


Figure 7. Increasing signal-to-noise ratio: blocking non-specific reactions.

Effect of Assay Method on Interpretation

The specificity of antibodies detected by a particular assay method should be considered when interpreting serologic results. The HAI is a very strain- or type-specific test because it detects only antibodies to the viral hemagglutinin, which frequently distinguishes between strains or serotypes within a virus group. On the other hand, the highly sensitive ELISA and IFA detect even small amounts of antibodies to most viral antigens, including those that are cross-reactive.

The effect of the test method on interpretation is demonstrated by recent findings regarding rodent parvoviruses. The prototype strains are minute virus of mice (MVM) and, in rats, Kilham rat virus (KRV) and Toolan's H-l. Traditionally, the HAI has been used to screen rodent colonies for parvovirus antibodies. As mentioned, antibodies detected by this method are strain-specific (see Table 2). KRV antiserum inhibits hemagglutination caused by KRV but not by H-l or MVM. Similarly, H-l and MVM antisera have HAI titers to homologous but not heterologous rodent parvovirus.

Table 2. Strain Specificity of HAI Test

	HAI TITER		
Antiserum	**RV**	**H1**	**MVM**
RV	160	-	-
H1	-	20,480	-
MVM	-	-	10,240

- = titer less than 20. Adapted from Siegl, Virol. Monographs, Vol. 15, 1976.

Consistent with a general switch to solid phase immunoassays, rodent parvovirus HAI have been largely replaced by the ELISA and IFA. Especially with IFA, Charles River and other laboratories have detected parvovirus antibodies in MVM and KRV/H-I HAI negative mouse and rat colonies, respectively. Because the strain-specific HAI results are negative, the IFA positive results are judged due to cross-reacting antibodies formed in response to a parvovirus strain or strains distinct from the prototype rodent parvoviruses, a rodent orphan parvovirus (ROPV).

ROPV antibody-positive colonies (i.e., HAI-negative but IFA-positive) are usually ELISA-negative. The IFA is more sensitive than the ELISA for detecting cross-reacting ROPV antibodies because of the antigen used in each test. The antigen in the IFA is virus-infected cells, which contain non-structural as well as structural viral proteins. A structural protein becomes part of the virus particle (i.e., virion); a non-structural protein does not. The parvovirus non-structural protein, NS-I, is antigenically conserved for different parvovirus strains. The crossreacting ROPV antibodies detected in the MVM and KRV IFA are probably specific for NS-I. In contrast, ELISA antigen is purified virus particles and contains little of the cross-reactive NS-I protein. Consequently, the ELISA, although very sensitive, misses cross-reacting antibodies detected by the IFA. A summary of our interpretation of rodent parvovirus serology results is shown in Table 3. Note that in rats, we have found some colonies that are usually HAI-negative but consistently ELISA as well as IFA-positive. We refer to this serologic diagnosis as ROPV "type 11." As this is sometimes seen with high KRV HAI titers, the distinction between it and KRV is quite tenuous.

Table 3. Rodent Parvovirus Serology

SPECIES	RESULT			INTERPRETATION
	HAI	ELISA	IFA	
RAT	-	-	-	NON-IMMUNE
	+	+	+	KRV OR H-1
	-	-	+	ROPV "TYPE 1"
	+/-	+	+	ROPV "TYPE 2"
MOUSE	-	-	-	NON-IMMUNE
	+	+	+	MVM
	-	-	+	ROPV

We have never observed any disease, pathology, or decrease in reproductive performance in association with seroconversion to ROPV. However, as ROPV has NOT been isolated or characterized, its effects on research are unknown.

Response to Unexpected Positive Results

Confirm results. Because results may be inaccurate, unexpected findings must be confirmed, especially those that are positive. This may involve testing additional animals or using alternative serologic tests (and/or other diagnostic methodologies such as pathology and bacteriology). Virus isolation and virus detection and identification by immunological methods or electron microscopy may be necessary.Become informed and act in a deliberate manner. Once results are confirmed, it is important to maintain perspective and avoid precipitous action. What is done about seropositive animals should be based on reference to the scientific literature. A particular agent, though present, may have no significant effect on the research at hand or the research of others.

Decide whether to keep the exposed rodents. Animals with a zoonotic infection should immediately be euthanized, decontaminated and disposed of safely. Because viruses often suppress the immune response (sometimes even after recovery from active infection), animals exposed to virus should not be used in delicate immunological research. Such animals may also be inappropriate if the agent has a tropism for the organ or tissue being studied; if it causes a persistent infection and may contaminate transplanted cell lines and other specimens derived from the host; or if the agent cannot be kept from spreading. LDV (lactate dehydrogenase-elevating virus) and MVM are examples of agents that cause persistent but latent infections. They have little obvious effect on mice but have been shown to be common contaminants of tumors transplanted into infected mice.

If recycling is necessary: it is crucial to consider whether or not a recurrence of the infection can be prevented. To reduce the likelihood of recurrence, possible sources of infection should be investigated (e.g, the animal vendor or transporter, wild rodents or contaminated biological materials), and improvements should be made in facilities and procedures. Isolators should be considered, especially in facilities with animals from many sources. Biological materials should be cultured for mycoplasma and examined for extraneous murine viruses by the mouse antibody production (MAP) test.

Euthanized animals should be sealed in bags and incinerated to prevent spread of virus throughout the facility. The animal room and its contents must be decontaminated, e.g., by autoclaving, paraformaldehyde gasing, and spraying or wiping surfaces with disinfectant. Some highly virucidal disinfectants are peracetic acid, commercial bleach, and ABQ Sterilant (Alcide Corporation). Virucidal disinfectants are especially important following adventitious infections with non-enveloped viruses (e.g., rodent parvoviruses) which are very resistant to inactivation.

If recycling is not necessary: measures should be taken to prevent spread of infection. Access to the infected colony should be limited, and all material removed from the area should be disinfected. It may be advisable to move the colony off-site, into a biocontainment area and/or into isolators. With enveloped viruses causing non-persistent infections (e.g., Sendai, the coronaviruses and pneumonia virus of mice), a 6-8 week moratorium on breeding and introduction of susceptible animals can often break the cycle of infection. During this period, all animals in the colony undergo active infection and recover. Any virus shed into the environment becomes non-infectious, as enveloped viruses are labile at room temperature. To ensure that the cycle has been broken, antibody-negative sentinels should be introduced after the moratorium and checked 3-6 weeks later. Colony offspring cannot be monitored because they will have maternal antibodies.

Conclusion

Adventitious infections with pathogenic viruses, bacteria and parasites adversely affect the suitability of rodents for research. Some agents indigenous to rodents can cause disease in people. To prevent infections, rodents are cesarean-derived and produced/maintained in isolators and barriers, but viral infections continue to occur. Routine monitoring for viruses is therefore necessary. The methodology most often employed is serologic testing for specific antibodies.

Valid serology results depend on the correct choice of assays and animals for testing, proper preparation and storage of samples to be tested, and precise preparation and performance of assays. No serologic test is always accurate. Results may be complicated by false positives and negatives due to many factors, and should therefore be interpreted cautiously. We believe it is imperative that unexpected positive findings be confirmed by using alternative assays and retesting more animals. Once infection is confirmed, further action should be based on its effects on research, other colonies and laboratory workers, and the likelihood of preventing reinfection after recycling.

As noted in the introduction, this Technical Bulletin was preceded by issues in Winter and Fall 1990 on health monitoring and serologic testing. The three provide a complete overview and may be requested from Charles River Laboratories.

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