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Improving Murine Health Surveillance Programs with the Help of On-site Enzyme-linked Immunosorbent Assay

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Timely and accurate detection of murine pathogens is essential in contemporary biomedical research. Cost, accuracy, and reproducibility of test results are frequent concerns when initiating an on-site serology program. This study was conducted to evaluate the advantages of on-site serology performed by enzyme-linked immunosorbent assay (ELISA) versus pathogen surveillance conducted off-site by a commercial vendor. We divided 92 sentinel mouse serum samples and tested them in parallel for a panel of 10 murine pathogens at our institution and by an off-site vendor. On-site testing was performed with commercially available test kits and according to the kit manufacturer's directions, whereas serum samples for off-site testing were prepared according to the vendor's specifications. Results from the 2 testing strategies were compared, and a good beyond-chance level of agreement was demonstrated by means of the kappa test ($\kappa = 0.86$). The turn-around time between sample preparation and results availability for on-site ELISA was 16 h versus 72 h for off-site testing. On-site ELISA demonstrated considerable cost reduction, ranging from 15.10% to 43.33% depending on the number of agents being tested. This study demonstrates the accuracy and time- and cost-effectiveness of on-site ELISA as well as its potentially valuable role in achieving more timely and efficient disease surveillance and control programs in contemporary biomedical research facilities.

Abbreviations: DLAM, University of California–Los Angeles Division of Laboratory Animal Medicine; ELISA, enzyme-linked immunosorbent assay; LCMV, lymphocytic choriomeningitis virus; MHV, mouse hepatitis virus; MPV, mouse parvovirus; MVM, minute virus of mice; OD, optical density units; PVM, pneumonia virus of mice;

The timely availability of health surveillance results with the least cost required is of paramount importance in rodent disease monitoring and control. At the University of California–Los Angeles Division of Laboratory Animal Medicine (DLAM), rodent disease monitoring has always been performed by enzyme-linked immunosorbent assay (ELISA) via a commercial vendor. With this system, serum samples were collected and diluted according to the vendor's specifications and shipped once a week to the external laboratory, not later than Tuesday, to ensure inclusion in the vendor's weekly assay runs. The turn-around time for the transmission of negative results from the vendor back to DLAM required a minimum of 3 working days from the time of mailing. For confirmatory testing of inconclusive or positive ELISA results, at least 1 additional working day was required for notification. Results for follow-up or additional serum submissions within the same week did not become available until after the next weekly run. In the face of an apparent outbreak or in disease situations that necessitate immediate intervention, quick test result availability and flexibility in test scheduling would be the order of the day. Moreover, with the upward animal population curve and the rising cost of off-site laboratory charges, conducting the tests on-site may be the better alternative. This study was therefore conducted to determine the merits of on-site ELISA in comparison with off-site ELISA in addressing the need for providing faster test results and reducing test costs while maintaining high levels of test specificity and sensitivity.

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Materials and Methods

Animals. We bled 92 sentinel mice (Crl:CD-1, Charles River, Wilmington, MA) by cardiac puncture immediately after euthanasia with carbon dioxide inhalation. These animals were 4 to 6 wk of age when first delivered to DLAM to form part of the Sentinel Program Protocol and were maintained in standard husbandry conditions (12:12-h light:dark cycle, room temperature of 20.0 to 22.2 °C, relative humidity of 35% to 70%, and free access to food and water) as approved by the Institutional Animal Care and Use Committee.[4] They were kept in barrier and conventional housing facilities and were provided with standard rodent formulated diet (Harlan, Madison, WI). Nonsurvival blood and tissue collection from each sentinel batch was performed every 3 mo for routine postmortem examination, serologic monitoring for certain viral and bacterial agents, and to check for ecto- and endoparasites. Each batch of sentinel animals is kept in the facilities for no more than 6 mo, according to the proportion of 1 sentinel cage for every 70 mouse cages in ventilated racks and 1 sentinel cage for every 42 cages in static racks.

Test sera. Individual serum samples from the sentinel mice were collected and immediately divided into 2 aliquots: 1 for on-site ELISA, and 1 for off-site ELISA.

On-site ELISA. Using commercially available ELISA test kits (Bartels, Carlsbad, CA) and following the manufacturer's recommendation,[1] we diluted each serum sample 1:50 in phosphate-buffered saline and tested for 10 pathogens, namely, Sendai virus, reovirus 3, mouse rotavirus, pneumonia virus

Table 1. Cost comparison for testing 92 serum samples for 10 individual agents, mouse parvovirus (MPV) screen (3 antigens), and 11-agent sentinel panel by means of off-site and on-site ELISA

Nature of expenditure	1-Agent Test (n = 920 tests)		MPV Screen (n = 276 tests)		11-Agent Test (n = 1012 tests)	
	Off-Site	On-Site	Off-Site	On-Site	Off-Site	On-Site
Technician time	1.5 h	13.5 h	1.5 h	6.5 h	1.5 h	14 h
Labor cost	$39.55	$355.94	$39.55	$171.36	$39.55	$369.08
ELISA testing cost	$7268.00	NA	$1962.36	NA	$5269.30	NA
ELISA test kit[a]	NA	$3750.00	NA	$1125.00	NA	$4125.00
Mailing	$30.00	NA	$30.00	NA	$30.00	NA
Laboratory supplies[b]	$47.63	$79.22	$47.63	$79.22	$47.63	$79.22
Total cost	$7385.18	$4185.16	$2079.54	$1375.67	$5386.48	$4573.30
% Savings	43.33%		33.84%		15.10%	
Cost/serum	$8.03	$4.55	$22.60	$14.95	$58.55	$49.71

NA, not applicable.
[a]Costs reflect purchase of 10 kits for the single-agent tests, 3 for the MPV screen, and 11 for the sentinel panel.
[b]Test tubes, pipette tips, serum vials, and latex gloves.

of mice (PVM), lymphocytic choriomeningitis virus (LCMV), *Mycoplasma pulmonis*, Theiler murine encephalomyelitis virus (TMEV), mouse hepatitis virus (MHV), minute virus of mice (MVM), and mouse parvovirus (MPV), Each test kit consisted of two 96-well ELISA plates and ready-to-use conjugates, substrates, stop solution, and wash buffers. The test plates were made up of removable antigen-coated strips (positive antigen control well) and removable strips without pre-coated antigen (negative antigen control well).

Test procedure. Indirect ELISA[10-12] was performed, and results were analyzed according to the ELISA kit manufacturer's recommendations.[1] Briefly, the main steps were: 1) pipeting of 100 µl diluted serum into each of the positive and negative antigen control wells and incubation at 37 °C for 45 min; 2) pipeting of 100 µl conjugate consisting of horseradish peroxidase-labeled antimouse immunoglobulin G into each test well and incubation at 37 °C for 45 min; 3) addition of 100 µl ready-to-use substrate indicator solution consisting of 2,2'-azino-di(3-ethyl-benzthiazoline-sulfonate-6) plus hydrogen peroxide into each well and incubation at room temperature (20 to 25 °C) for 30 min; 4) washing of plates 5 times with wash buffer between steps 1 to 3 with the help of an ELISA strip washer (Captia Washer, Trinity Biotech, Carlsbad, CA); 5) reading of the colorimetric reaction absorbance by use of a strip reader (Captia Reader, Trinity Biotech) at a wavelength of 405 nm; and 6) addition of 25µl stop solution into each well if the reaction could not be read right away.

Data analysis. The reaction of each serum sample was classified as positive or negative against a specific agent depending on the ELISA kit manufacturer's predetermined cut-off value.[1] The sample absorbance on the negative antigen well was subtracted from the same sample absorbance on the positive antigen well, and a positive reaction was denoted by an absorbance difference of at least 0.300 optical density units (OD). According to the ELISA kit manufacturer's recommendations, acceptance of test results from any given test plate was dependent on the fulfillment of the following set of criteria: 1) the negative control serum should produce an absorbance of no more than 0.250 OD on the positive antigen well at 405 nm; 2) the positive control serum should produce an absorbance of at least 0.600 OD on the positive antigen well at 405 nm; and 3) both positive and negative control sera should produce an absorbance of no more than 0.250 OD on the negative antigen control well at 405 nm.

Reproducibility of test results. The reaction of each serum sample against each of the 10 pathogens used in this study was tested in 2 separate assay runs. Each serum reaction was determined with the help of the previously mentioned cut-off value of 0.300 OD.

Off-site ELISA. Each serum sample was diluted 1:5 in phosphate-buffered saline in accordance with the external laboratory's specifications,[2] packed, and shipped via a standard overnight delivery service for the detection of the 11-agent UCLA mouse panel which consisted of the 10 previously mentioned disease agents plus *Ectromelia* virus.

Comparison of on-site and off-site ELISA. The 2 testing strategies were compared based on the following parameters: 1) turn-around time, or time spent from sample preparation to the availability of test results; 2) the kappa index (κ) for demonstrating the beyond-chance level of agreement between on-site and off-site test results; and 3) the costs of testing 1 serum sample for 10 single agents, a panel of 11 agents, and an MPV screen consisting of MVM, MPV viral capsid protein 2, and MPV nonstructural protein 1. The testing costs were computed based on laboratory charges, shipping, labor, and material cost and are presented in Table 1.

Computation and interpretation of the kappa index. With off-site ELISA as the reference test, each on-site serum reaction was classified as true positive, true negative, false positive, or false negative and shown in a 2×2 contingency table.[3]

The kappa index was computed and interpreted in light of the values associated with perfect agreement ($\kappa = 1.0$), no agreement ($\kappa = 0.0$), and average, beyond-chance level of agreement ($\kappa = 0.4$ to 0.5).[3,6] The standard error and 95% confidence limits were determined by applying the formula for Cohen's unweighted kappa.[3,9]

Cost and savings analysis. Using the DLAM vouchers from March 2002 through February 2004, we determined the total annual count of mouse sera submitted for serology and the corresponding ELISA test scheme from March 2002 to February 2003 and from March 2003 to February 2004 (Figure 1). The cost of MPV screening as well as testing for single and 11 agents during these time frames was recorded. The total expenses for off-site ELISA was calculated for the period from 2003 to 2004 and compared to on-site ELISA to demonstrate possible savings if on-site ELISA was performed during the same time frame. The future cost and savings projection was made based on the 2003 to 2004 ELISA test trend, 3% cost inflation, and 219 additional sentinel animals required for the opening of 3 new facilities that were designed to hold at least 15,000 mouse cages (Figure 2).

All tables, graphs, and computations performed in this study were generated with Excel 2004 (Microsoft, Redmond, WA).

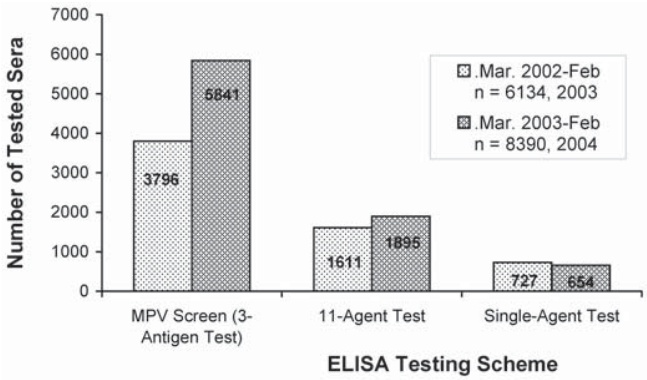

Figure 1. Total number and classification of ELISA tests performed from March 2002 to February 2004. MPV screen represented the highest number of submissions during this 2-y study period, followed by the 11-agent panel test and lastly by the single-agent test.

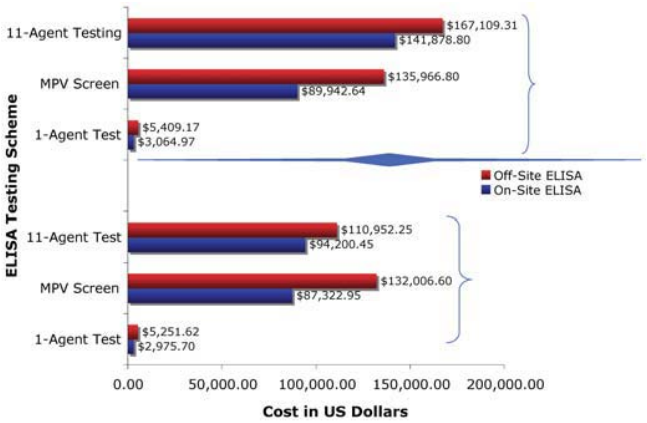

Figure 2. On-site and off-site ELISA annual and projected costs. The values below the horizontal line represent the individual test scheme cost per serum multiplied by the total number of ELISA tests conducted from March 2003 to February 2004 (refer to Figure 1). The values above the horizontal line are based on the annual costs for March 2003 to February 2004 and reflect the projected increase in sentinel animals as new rodent facilities are opened plus an allowance for a 3% cost inflation. These figures suggest a foreseeable savings of at least $73,598.88 (23.86%) with the use of on-site ELISA.

Results

Reproducibility of on-site ELISA test results. By applying the recommended 0.300 OD cut-off value, the same serologic reaction was observed from each serum sample when tested in 2 separate assay runs (Assays A and B) against the 10 disease agents. In both runs, 16 samples reacted positively to MPV, 3 to MHV, 2 to rotavirus, and 1 to MVM, whereas all samples remained serologically negative to LCMV, *M. pulmonis*, PVM, reovirus 3, Sendai virus, and TMEV.

Turn-around time for testing 92 sera. With the type of ELISA washer and reader used, the on-site individual testing of 92 sera for 10 agents required 16 h, or 8 h per 46 sera from sample preparation and dilution to availability of test results. This value was reduced further to 13.5 h for testing 92 sera if a full-plate ELISA washer and reader were used. Off-site individual testing, in comparison, took at least 72 h before the results were made available; this value did not include the 1.5 h of technician time at DLAM for sample dilution, packaging, and mailing (Table 1).

Computation of the kappa index. This test measures the beyond-chance level of agreement between off-site and on-site ELISA test results. Of the 920 serum reactions observed, 895 were classified as true negatives (for PVM, LCMV, TMEV, reovirus, *M. pulmonis*, and Sendai virus). Another 3 and 16 samples reacted positively for MHV and MPV both on-site and off-site, respectively, thereby giving a total of 19 specimens classified as true positives. On-site testing detected 1 MVM-positive and 2 rotavirus-positive samples that remained negative off-site and were thus designated as false positives relative to the reference test used. In addition, 3 samples reacted positively for MPV off-site but not on-site and therefore were tagged as false negatives when compared to the reference test. The computed kappa index is $\kappa = 0.86$ (standard error = 0.057; 95% confidence interval = 0.75 to 0.97). According to the criteria for κ interpretation,[3] this value suggests a good beyond-chance level of agreement between on-site and off-site ELISA results was observed.

Labor, test, and material costs. The labor cost for sample preparation and shipment, based on the $26.36/h salary scale of a Staff Research Associate II inclusive of benefits, was $39.55 for off-site ELISA (Table 1). For on-site ELISA, this cost varied from $171.36 to $369.08, depending on the number of pathogens tested. The prevailing off-site laboratory charge for each serum sample at the time of study was $7.90 for a single-agent ELISA, $21.33 for MPV screen, and $57.28 for an 11-agent ELISA. For on-site testing, the cost of each test kit containing two 96-well plates was $375.00 and was enough to test 92 serum samples. The costs of testing 1 serum sample on-site for 1 pathogen, MPV screen, and 11 pathogens were $4.55, $14.95, and $49.71, respectively; in comparison, off-site testing cost $8.03, $22.60, and $58.55, respectively.

Cost and savings analysis. The total number of rodent sera submitted to the off-site vendor in the period of March 2002 to February 2003 was 6134, which rose to 8390 in the following year (Figure 1). Parvovirus screening was the most frequently conducted testing scheme, whereas single-pathogen testing was least frequent during the 2-y survey period. The total amount spent by DLAM for off-site testing from March 2003 to February 2004 was $248,210.47 (Figure 2). This value was obtained by multiplying the test cost for each serum sample shown in Table 1 with the corresponding total number of ELISA testing scheme shown in Figure 1. Calculation of in-house testing cost for the same time frame would have been $184,499.10, with a total test cost difference of $63,711.37, or a savings of 25.67% (Figure 2). If the same disease trend continues and as new rodent facilities on campus are opened, a savings of at least $73,598.88 (23.86%) is foreseen. This figure includes the addition of at least 219 sentinel animals for 3 new facilities and an allowance for a 3% cost inflation (Figure 2).

Discussion

This study was conducted to demonstrate the merits of conducting ELISA within the UCLA-DLAM premises, with due consideration not only for its cost-effectiveness but also for the reproducibility of test results as well as the amount of time required to complete the assays. The rodent sentinel program at DLAM routinely screens for antibodies against at least 11 viral and bacterial agents in the barrier facilities and against at least 19 pathogens in the defined-flora facilities. Disease surveillance at DLAM has always been dependent on the serology reports provided by a commercial vendor, where serum samples were regularly sent and tested for murine pathogens. The transport of serum samples from DLAM to the vendor laboratory entailed spending additional time and laboratory resources (Table 1). With the rising rodent population on campus (currently 40,000 cages of mice) and increasing demand for a more timely availability of test results, the idea of performing ELISA on-site was

brought forth.

ELISA is an indirect diagnostic test whose efficiency is evaluated based on its ability to identify an animal that has been exposed to a disease agent as test positive and a nonexposed animal as test negative, otherwise known as sensitivity and specificity, respectively.[3,6] A standard diagnostic procedure that directly demonstrates the causative agent, for example by culture or polymerase chain reaction, usually serves as the 'gold standard' for comparing the sensitivity and specificity of 2 ELISA techniques.[3] Previous studies, however, showed that ELISA was more sensitive than the standard culture method in detecting infections or exposure to infectious agents.[10-12] In the present study, the same serologic reaction was observed from each serum when tested in 2 different assay runs, thus demonstrating the reproducibility of on-site ELISA test results. Statistical tests to demonstrate the reliability and validity of results obtained from the ELISA kits used were not done in this study because these kits were already commercially available and therefore presumed to have been tested for precision prior to being approved for distribution. One problem encountered during the conduct of this study was the unavailability of test kits for *Ectromelia*, which was a component of the basic 11-agent UCLA panel, thus limiting the number of on-site ELISA test agents to 10. For purposes of comparison, the computation for labor and material cost for an 11-agent on-site ELISA was adjusted correspondingly (Table 1).

In comparing the results of on-site ELISA with those of off-site ELISA, we used the kappa test to demonstrate whether the level of agreement between the 2 ELISA testing methods was real and not merely due to chance.[3] In this evaluation, off-site ELISA served as the reference test for classifying each on-site ELISA serum reaction as true positive, true negative, false positive or false negative since this method has been applied as the routine detection method for years at DLAM. One limitation of this strategy was the possibility that the methodology and reagents used in on-site ELISA may have rendered the test greater sensitivity and specificity, thereby qualifying the so-called false-positive and false-negative results as real reactions. No confirmatory test was done to evaluate all the positive reactions detected by on-site ELISA, in contrast to what was done for off-site ELISA, where all the positive or inconclusive results were confirmed by other serological tests like the hemagglutination inhibition and indirect fluorescent antibody tests. Although another strategy using animal populations with known infection or exposure status may have helped in determining the accuracy of both test methods, preference was given to test sera from the 'real world,' that is, from actual sentinel animals that were used to indirectly monitor animals housed in barrier as well as conventional facilities. Despite the occurrence of reactions that were classified as false-positives and false-negatives in this study, the kappa index obtained (0.86) was still indicative of a good beyond-chance level of agreement between off-site and on-site ELISA. This finding suggests that ELISA test results obtained from on-site testing are just as accurate as those obtained from external laboratories.[3]

The discrepancy observed in the 2 rotavirus, 1 MVM, and 3 MPV test results can be explained mainly by interlaboratory variation that can be brought about by differences in reagent concentrations and volumes, plate incubation times, and prevailing ambient temperature. The sensitivity and specificity of any given ELISA test system are highly dependent on the selected positive–negative threshold or cut-off, which invariably affect the percentage of false-positive and false-negative results. The predetermined 0.300 OD cut-off used in this study is an empirical value that has been widely used in different ELISA set-ups. Depending on the goals of the study, this value can be increased to improve the specificity or decreased to improve detection rate, thereby decreasing the chance of getting false-negative or false-positive results.[3,10]

The mode of antibody activity expression presents another major difficulty in comparing results between laboratories.[10] Both off-site and on-site ELISA use 'corrected' OD values, where the control antigen OD value is subtracted from the sample OD value to compensate for binding of antibody to host cell components. This system is commonly used in virologic systems, but these values are of little use for intra- and interlaboratory comparisons.[10] A more plausible expression of antibody activity for intra- and interlaboratory comparison would be the testing of each sample OD against a standard curve generated from a panel of 4 to 5 positive reference sera and must be available for use by the laboratories concerned.[5,10]

A greater number of rodent serum samples were submitted to the commercial vendor for ELISA during the 2nd year of the 2-y survey from 2002 to 2004 (Figure 1). This trend is expected to continue as more facilities become operational in the future and as the number of transgenic and knockout mice increase at a rapid pace. According to the number of samples tested (8,320) and the amount spent during the fiscal year 2003 to 2004 for outside testing ($248,210.47) as well as the estimated 25.67% cost savings (equivalent to $63,711.37) if ELISA were performed at DLAM for that year (Figure 2), on-site ELISA proves to be more cost-effective than off-site ELISA. The projected 23.86% annual savings of $73,598.88 after February 2004 was a conservative estimate. Considered in this computation were the increased number of sentinel animals required and a 3% allowance for cost inflation. The cost and savings for parvovirus screening, which was consistently the most frequently conducted testing scheme during the 2-y survey period, is yet to be included. Compared with off-site testing, on-site sentinel testing for a panel of 11 pathogens incurred a savings of 15.10% (Table 1). For parvovirus screening, the savings were higher, at 33.84%. If this testing scheme were to form part of the projected annual savings estimate and if the same disease trend continues, more savings are expected to come with on-site performance of ELISA.

In the cost analysis (Table 1), the energy cost needed to run the ELISA strip reader and washer was not included because these equipment models did not represent what was actually being used in laboratories where large numbers of samples usually are processed. If a 96-well full-plate ELISA washer and reader were used in the study to process the 92 serum samples, 30 min would have been the maximum operation time for each machine (this value includes calibration, priming, and rinsing) and translates to a power consumption of 0.25 kW × h or a present-day cost of $0.03, according to our November 2005 utilities statement. These machines are run by a low power source (<250 W) and require a short period of time to complete the task, that is, 12 s to read a plate and 6 min to wash 10.8 plates.[7,8] When the computed energy cost was added to the total cost analysis shown in Table 1, the cost of testing per serum, which was rounded to 2 decimal places, remained the same. More importantly, the cost of utilities was not included in the analysis because in large academic institutions like UCLA and University of Florida, this expenditure is shouldered by the central administration and not by the individual departments or divisions and consequently does not contribute to the investigators' per diem.

The main difference between barrier and conventional housing facilities is the absence of cage-changing hoods in the conventional facilities. The seropositive reactors detected in the

present study were obtained mostly from the conventional housing facilities, but this result did not necessarily mean a lower disease prevalence in the barrier facilities. Despite enforcement of strict barrier rules, the prevention and control of disease outbreaks may continue to be a challenge because of other factors that are beyond control of the veterinary staff, such as the active exchange of animals between investigators.

With the on-site performance of ELISA, further improvements in rodent health monitoring are foreseen. In contrast to the fixed schedule for sample submission to and availability of results from the outside vendor, on-site assays can be scheduled and conducted any time, with flexibility. In addition, because the turn-around time is shorter, on-site ELISA becomes a vital surveillance tool for monitoring outbreaks of highly infectious diseases like mouse hepatitis virus. Although a positive indirect ELISA result indicates exposure to a pathogen that may have been in the facility for weeks or months, prompt detection in a closely followed surveillance program is still valuable in monitoring the course of an infection, making decisions on managing the affected colonies, and instituting disease control measures. This information especially serves as a useful guide for the animal care personnel and investigators as they go from one facility to another with or without their animals. Flexibility in test scheduling and quick turn-around time are beneficial in updating the health status of a room or a rack where animal movement into or out of that room is indicated. The value of on-site ELISA therefore lies not only in the annual cost savings but also in the fast and timely availability of test results for a more efficient disease prevention and control strategy.

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