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First Trial in the Developmental Phase of the “Performance Evaluation Program” Based on the ICLAS Animal Quality Network Program: Self-Assessment of Microbiological Monitoring Methods Using Test Samples Supplied by ICLAS

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Abstract: The first trial in the developmental phase of the “Performance Evaluation Program” based on the new programs of the International Council for Laboratory Animal Science (ICLAS) was carried out. ICLAS supplied test samples to each diagnostic laboratory for self-assessment of microbiological monitoring methods. We found that 1 mouse serum sample was positive for mouse minute virus and another was positive for *Mycoplasma pulmonis* antibodies, and 1 rat serum sample was positive for Sendai virus antibody. Mouse parvovirus was detected from mouse spleen and mesenteric lymph node homogenate, and *Helicobacter* spp. were detected from mouse feces. *Corynebacterium bovis* was isolated from a mouse skin sample. These results were in agreement with those notified by the ICLAS after the trial. After this trial, the program will eventually be made available to all diagnostic laboratories willing to participate in it.

Key words: ICLAS, microbiological monitoring

In 1979, the International Council for Laboratory Animal Science (ICLAS) Monitoring Center was established in Japan on the basis of the ICLAS-coordinated Monitoring Center System proposed by the 7th International Committee on Laboratory Animals (ICLA) General Assembly [11]. In 2006, the ICLAS Governing Board approved the replacement of the Monitoring and Reference Center Program with a new more international program called the ICLAS Animal Quality Network, to serve as an international reference in the field

of high quality laboratory animal models [14]. The objectives of this new initiative are to develop programs that will achieve the ICLAS aim of improving the quality of animals used in research and to organize workshops of interest [12]. At least 1 scientific workshop will be organized every 4 years, so as to coincide with the ICLAS General Assembly. At the 2006 ICLAS meeting, we were informed that our center (Central Institute for Experimental Animals; CIEA, Kanagawa, Japan) would be a member of the network. The members of this net-

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work currently include CIEA; Centro Multidisciplinar para Investigação Biológica, Universidade Estadual de Campinas (CEMIB-UNICAMP; São Paulo, Brazil); Microbiological Diagnostic, German Cancer Research Center (Heidelberg, Germany); Research Animal Diagnostic Laboratory (RADIL; University of Missouri MO, USA); Research Animal Diagnostic Services, Charles River Laboratories (CRL; MA, USA); The Jackson Laboratory (ME, USA); QM Diagnostics, Radboud University Medical Centre Nijmegen (Gelderland, Netherlands); and the Institute for Laboratory Animal Research (ILAR; Washington, D.C., USA) [9].

In 2007, the “Performance Evaluation Program” (PEP), which was based on the ICLAS Animal Quality Network Program, was presented at the ICLAS meeting in Cernobbio, Italy [13]. The PEP is designed to assist animal diagnostic laboratories in assessing the sensitivity and specificity of their health-monitoring assays [14] and to help assure the quality of laboratory animals used in research. The first trial in the developmental phase of the program, which is described here, was organized by RADIL and CRL and conducted by a closed core group consisting of members of the network prior to the program being made available to all diagnostic laboratories that wish to participate in the PEP. Before the tests were performed by ICLAS, an inventory of samples and pathogens was sent to the participating laboratories and the samples were tested for the listed pathogens. Normal samples were also included. In this paper, we describe the program, details of the trial, and the results; further, we discuss the first trial to ensure the universal application of the program.

Pathogens listed in the inventory sent before the tests were conducted are shown in Table 1. They are as follows: mouse parvovirus (MPV), mouse minute virus (MMV), mouse hepatitis virus (MHV), ectromelia virus, lymphocytic choriomeningitis virus (LCMV), Sendai virus, *Mycoplasma pulmonis*, *Helicobacter* spp., *Pasteurella pneumotropica*, *Salmonella* spp., *Corynebacterium bovis*, *Staphylococcus aureus*, rat parvovirus (RPV), Kilham rat virus (KRV), rat minute virus (RMV), and sialodacryoadenitis virus (SDAV).

The tests were performed using routine methods used at CIEA, as described below.

C. bovis is not among the routine test items used at

Table 1. Specimen inventory notified in advance

Species	Agents	Sera	Microbial specimens
Mouse	Normal	○	
	MPV	○	○
	MMV	○	○
	MHV	○	○
	Ectromelia virus	○	○
	LCMV	○	○
	Sendai virus	○	○
	<i>Mycoplasma pulmonis</i>	○	○
	<i>Helicobacter</i> spp.		○
	<i>Pasteurella pneumotropica</i>		○
	<i>Salmonella</i> spp.		○
	<i>Corynebacterium bovis</i>		○
	<i>Staphylococcus aureus</i>		○
Rat	Normal	○	
	RPV	○	○
	KRV	○	○
	RMV	○	○
	SDAV	○	○
	Sendai virus	○	○
	<i>M. pulmonis</i>	○	○

MPV: Mouse parvovirus; MMV: Mouse minute virus; MHV: Mouse hepatitis virus; LCMV: Lymphocytic choriomeningitis virus; RPV: Rat parvovirus, KRV: Kilham rat virus; RMV: Rat minute virus; SDAV: Sialodacryoadenitis virus. Antibodies against the agents shown in Table 4 were tested by ELISA using serum samples. Viruses and *Helicobacter* spp., and other bacteria in microbial specimens (Table 4) were tested by (RT)-PCR and the culture method, respectively. The inventory was sent from ICLAS in advance.

CIEA, and isolation and identification of this bacterium was performed as previously described [23]. At CIEA, serological diagnosis is performed using enzyme-linked immunosorbent assays (ELISA) and/or indirect immunofluorescent assays (IFA). ELISA and IFA were performed as previously described [3, 18, 21]. The sera were diluted to 1:40 for ELISA and 1:10 for IFA with saline containing 0.1% sodium azide. In the case of ELISA, horseradish peroxidase (HRP)-conjugated protein A (Wakamoto Pharmaceutical Co. Ltd., Tokyo, Japan) and HRP-conjugated anti-rat immunoglobulin (Ig) G (Wakamoto) were used to detect mouse and rat antibodies, respectively. Colorimetric activity was measured at 490 nm on a microplate reader (Benchmark Plus™ Microplate Spectrophotometer, Bio-Rad Laboratories Inc., Tokyo, Japan). An optical density (OD) of 0.3 or higher was regarded as positive. In the case of IFA,

Table 2. Viral and bacterial strains, and cell lines used for propagation of the organisms

Agents	Strains	Methods	Cell lines	References
MPV	UT (VP2 gene)*	ELISA	<i>E. coli</i> (BL21)	20
MMV	Prototype (p)	ELISA/IFA	A9 cells	20
MHV	S	ELISA	DBT cells	17
	Nu67	IFA	DBT cells	
Ectromelia virus	Vaccinia virus Lederle strain	ELISA/IFA	Hela cells	2
LCMV**	WE strain	IFA	L cells	9
Sendai virus	MN strain	ELISA/IFA	LLC-MK2 cells	17
<i>M. pulmonis</i>	m53	ELISA/IFA	BHK-21 cells	8, 19
KRV	Kilham(RV-13)(ATCC VR-235)	ELISA/IFA	C6 cells	20
SDAV	MHV-Nu67 strain***	ELISA/IFA	DBT cells	17

*Recombinant antigen. **IFA antigen plates were obtained from Nagasaki University (Dr.Hiroshi Sato).

***MHV-Nu67 antigen cross-reacts with SDAV. ELISA antigen was prepared as shown in the references.

fluorescein isothiocyanate (FITC) anti-mouse IgG (MBL Co. Ltd., Nagoya, Japan) and FITC anti-rat IgG (ICN Pharmaceuticals Inc., Aurora, OH, USA) were used. The bacterial and viral strains and cell lines used for ELISA and/or IFA are listed in Table 2.

Mouse feces and skin swabs were cultured on 5% horse blood agar (Eiken Chemical Co. Ltd., Tokyo, Japan), deoxycholate-hydrogen sulfide-lactose (DHL) agar (Eiken), and egg yolk-salt agar (Eiken), and then incubated at 37°C for 24 h (for DHL) or 48 h (for other methods) under aerobic conditions. Colonies observed on the agar plates were identified on the basis of morphology, Gram staining, and biochemical testing using ID Test SP-18 (Nissui Pharmaceutical, Co. Ltd., Tokyo, Japan) or Api 20 NE (bioMerieux Japan Ltd., Tokyo, Japan).

The reverse transcriptase (RT)-polymerase chain reaction (PCR) method was used for the molecular detection of pathogens from mouse spleen and mesenteric lymph node (MLN) homogenate, and mouse feces. RNA and DNA were extracted using RNABee™ (Tel Test Inc., Friendswood, TX, USA) and MagExtractor® (Toyobo Co. Ltd., Tokyo, Japan), respectively, according to the manufacturers' instructions.

RT-PCR was performed as previously described [6]. The PCR products were electrophoretically separated on 4% agarose gel (AMRSCO®, Solon, OH, USA), stained with ethidium bromide, and visualized under ultraviolet (UV) light. Primer sequences and PCR product sizes are given in Table 3. The PCR primers for ectromelia virus used in this study were designed at our institute.

Viral or bacterial DNA or RNA (listed in Table 2) was used as the positive control for (RT-)PCR.

The samples, including 2 tubes of mouse serum and a tube each of rat serum, mouse spleen and MLN homogenate, mouse feces, and mouse skin swab, were received on November 8, 2007. The test samples (6 tubes in total) were packed in a styrene foam box (37 × 33 × 30 cm), with dry ice and shipped on November 5, 2007 from CRL by World Courier®. The tubes were intact on arrival. The volumes of the samples in the tubes were in agreement with the values in the list. The samples are listed in Table 4. The numbers of the samples are different from the numbers listed on the ICLAS homepage [14].

Mouse and rat sera were diluted 40-fold before use in the antibody test. Mouse feces were applied to agar plates using sterile cotton swabs. As described above, DNA and/or RNA were extracted from the mouse spleen and MLN homogenate, and mouse feces for (RT-)PCR.

As shown in Table 5, antibodies against MMV and *M. pulmonis* were detected in samples Nos. 7-1 (mouse serum) and 7-2 (mouse serum), respectively, and the antibody against Sendai virus was detected in sample No. 7-3 (rat serum) by using ELISA. MPV and *Helicobacter* spp. were detected in samples Nos. 7-4 (mouse spleen and MLN homogenate) and 7-5 (mouse feces), respectively, by using PCR. *C. bovis* was cultured from sample No. 7-6 (mouse skin swab). No other pathogens listed in the inventory were observed in this study. The results were in agreement with those notified by ICLAS after the tests.

Table 3. Primer sequences and (RT)-PCR product sizes

Agents*	Target gene	Primer	Sequences (5' to 3')	Product size (bp)	References
MPV/MMV	NS1	F R	ACCTCACCAGAAGACTGGATG CCAACATTACCAACTGCTTGTG	371	1, 20, 27
MPV	VP1/2	F R	GCAGCAATGATGTAAGCTGAAGCT CCATCTGCCTGAATCATAGCTAA	260	1, 20, 27
MMV	VP1/2	F R	GATGCTAATGCTTGGGGAG GGGTAGAAACCAAGTGTTC	263	1, 20, 27
MHV		reverse 1st F 1st R nested F nested R	random primer ATGTCTTTTGTTCCTGGGCA TTACACATTAGAGTCATCTTCT ATTCTACTAGGTTTGCGCC CTGCTTGTTTGGAGTCCTCT	318	4
Ectromelia virus	IFN γ R	F R	TATTATCCCTTCAGTGACCG GATAAAAATAGTTTAGAGAG	877	**
LCMV	NP	reverse F R	random primer TCCATRAGWGCACAGTGYGGGGTGAT GCATGGGARAAAYACRACAATTGAYC	590	9
Sendai virus	NP	reverse 1st F 1st R nested F nested R	random primer TGAAGTTATACAGGAT CCAGCACAATCCAGACTTGGAC TCCAGACCCTTTGCTTTGC ATTTGACATCGGCGTTTACTCCG	334	24
<i>Helicobacter</i> spp.	16S rRNA	1st F 1st R nested F nested R	CTATGACGGGTATCCGGC CTCACGACACGAGCTGAC AGGGAATATTGCTCAATGGG TCGCCTTCGCAATGAGTATT	350	5

*The agents in microbial specimens were tested by (RT)-PCR. **Primer sequences were designed at our institute.

Table 4. Samples sent from CRL*

ID number	Samples	Description	Test for	Quantity
7-1	Serum	Mouse serum, diluted 5-fold in PBS	Microbial antibodies	250 μ l
7-2	Serum	Mouse serum, diluted 5-fold in PBS	Microbial antibodies	250 μ l
7-3	Serum	Rat serum, diluted 5-fold in PBS	Microbial antibodies	250 μ l
7-4	Organs	Mouse spleen and mesenteric lymph node homogenate	Viruses	1 ml
7-5	Feces	Mouse feces	Bacteria	150 μ l
7-6	Swab	Mouse skin swab	Bacteria	1 ml

*The test samples were packed in a box (37 $\times$ 33 $\times$ 30 cm) with dry ice, and sent from Charles River Laboratories, Wilmington, MA, USA.

A trial in the developmental phase of the PEP was successfully performed at ICLAS and CIEA. Samples were received on the expected date; the boxes contained a sufficient volume (approximately 80%) of dry ice. The samples were maintained in a completely frozen state, no tubes were broken, and the test results were conclusive (false-positive reactions were not observed).

MPV has become one of the most prevalent pathogens in laboratory animal facilities in the USA [16], but this virus is not common in Japan [7]. In conventional laboratory mouse and rat facilities in Japan, *Staphylococcus aureus*, intestinal protozoa, *Pseudomonas aeruginosa*, and *M. pulmonis* have been observed, while the most common pathogens in Japan are MHV and *Pasteurella*

Table 5. Results of specimens from ICLAS

Organisms	Methods	Sample No.					
		7-1 Mouse serum	7-2 Mouse serum	7-3 Rat serum	7-4 Mouse MLN, spleen	7-5 Mouse feces	7-6 Mouse skin swabs
MPV	ELISA	Neg.	ND	ND	ND	ND	ND
	PCR	ND*	ND	ND	Pos.	ND	ND
MMV	ELISA/IFA	Pos./Pos.	Neg./Neg.	ND	ND	ND	ND
	RT-PCR	ND	ND	ND	Neg.	ND	ND
MHV	IFA	Neg.	Neg.	Neg.(SDAV)	ND	ND	ND
	RT-PCR	ND	ND	ND	Neg.	Neg.	ND
Ectromelia virus	IFA	Neg.	Neg.	ND	ND	ND	ND
	PCR	ND	ND	ND	Neg.	ND	ND
LCMV	IFA	Neg.	Neg.	ND	ND	ND	ND
	RT-PCR	ND	ND	ND	Neg.	ND	ND
Sendai virus	IFA	Neg.	Neg.	Pos.	ND	ND	ND
	PCR	ND	ND	ND	Neg.	ND	ND
KRV	IFA	ND	ND	Neg.	ND	ND	ND
<i>M. pulmonis</i>	IFA	Neg.	Pos.	Neg.	ND	ND	ND
<i>Helicobacter</i> spp.	PCR	ND	ND	ND	ND	Pos.	ND
<i>P. pneumotropica</i>	Culture	ND	ND	ND	ND	Neg.	Neg.
<i>Salmonella</i> spp.	Culture	ND	ND	ND	ND	Neg.	Neg.
<i>C. bovis</i>	Culture	ND	ND	ND	ND	Neg.	Pos.
<i>S. aureus</i>	Culture	ND	ND	ND	ND	Neg.	Neg.
RPV**		ND	ND	ND	ND	ND	ND
RMV**		ND	ND	ND	ND	ND	ND
Results***		MMV	<i>M. pulmonis</i>	Sendai virus	MPV	<i>Helicobacter</i> spp.	<i>C. bovis</i>

*ND: Not done. **Not available. ***Results obtained in this study. Serum samples (7-1, -2, and -3) were tested for pathogens shown in the first column by ELISA. The results were confirmed by IFA test, and microbial specimens (7-4, -5, and -6) were tested for pathogens by PCR (*Helicobacter* spp.) and the culture method (other bacteria). For antibodies against mouse parvoviridae, including MPV and MMV, sera were screened using MMV antigen which cross-reacts with both viruses. Then, MMV and MPV were detected using MPV recombinant VP2 antigen. For viral RNA detection of MVM and MPV, the most suitable organs for detection of the virus are MLN and spleen [25]; feces and skin swabs were not tested. *M. pulmonis* was not tested using microbial specimens (7-4, -5, and -6) because the respiratory organs were not included in the specimens. Ectromelia virus was not tested using mouse skin swab because the samples were not tissue samples but swab. The results observed in this study showed that 7-1, -2, and -3 were positive for MMV, *M. pulmonis*, and Sendai virus antibody, respectively, and 7-4, -5, and -6 were positive for MPV, *Helicobacter* spp., and *C. bovis*, respectively. These results were in agreement with results notified by ICLAS after the test.

pneumotropica [15]. MHV and *M. pulmonis* are enzootic to Korea [26]. When selecting pathogens for the PEP, the current microbiological status in each country should be considered, and the common pathogens in each country should be included in test samples. Sharing of such information within the Animal Quality Network can benefit the selection of suitable pathogens for the test samples.

Each testing laboratory has already established its own

monitoring system (methods) for testing; hence, samples should be adapted to each system. This is because the purpose of the PEP is not to check the ability of testing laboratories to analyze the samples but to enable them to assess their own systems. One idea is that, especially for antibody tests, these laboratories can request a sample volume before performing confirmatory tests such as the western blot assay.

C. bovis causes hyperkeratotic dermatitis in athymic

nude mice [23]. Outbreaks of the disease were reported in Italy [22]. RPV and RMV infections in rats had been previously reported [25]. Based on our monitoring experiences, outbreaks of these pathogens in animal facilities are not common in Japan (data not shown). Therefore, we should prepare a system to screen imported animals for these viruses. In this trial, ELISA, IFA, culture, and (RT)-PCR methods were used. In the future, a self-assessment program for endoparasites and pathological testing should be included because these tests require considerable skill as compared to other tests such as the ELISA antibody test, which can be performed using a commercial kit.

This is the first trial in the developmental phase of the PEP. After the program is made available to all diagnostic laboratories, the laboratories may detect problems with their monitoring systems such as those concerning the sensitivity of the methods and the selection of the strains from which antigens are derived. To solve these problems, a follow-up program including technical meetings, a consultation system, or a training program may be required. Currently, the PEP is focused on testing laboratories for laboratory animals. In the future, in addition to the self-assessment program, an educational program for monitoring that focuses on both testing laboratories and other users of laboratory animals may also be required.

References

1. Besselsen, D.G., Besch-Williford, C.L., Pintel, D.J., Franklin, C.L., Hook, Jr. R.R., and Riley, L.K. 1995. *J. Clin. Microbiol.* 33: 2859–2863.
2. Buller, R., Bhatt, P., and Wallace, G. 1983. *J. Clin. Microbiol.* 18: 1220–1225.
3. Goto, K., Itoh, T., Takakura, A., Kunita, S., Terada, E., and Kagiya, N. 1991. *Jikken Dobutsu* 40: 231–233.
4. Goto, K., Nozu, R., Kunita, S., Terada, E., and Itoh, T. 1995. *Exp. Anim.* 44: 159–161.
5. Goto, K., Ohashi, H., Takakura, A., and Itoh, T. 2000. *Curr. Microbiol.* 41: 161–166.
6. Goto, K., Takakura, A., Yoshimura, M., Ohnishi, Y., and Itoh, T. 1998. *Lab. Anim. Sci.* 48: 99–102.
7. Hayashimoto, N., Ishida, T., Yasuda, M., Ueno, M., Kameda, S., Shinohara, H., Goto, K., Takakura, A., and Itoh, T. 2005. pp. 49. In: 58th AALAS National meeting abstract.
8. Horowitz, S.A. and Cassell, G.H. 1978. *Infect. Immunol.* 22: 161–170.
9. ICLAS Animal quality network. 2006. Performance Evaluation Program for Diagnostic Laboratories (PEP). [Online] <http://www.iclas.org/Networkmembers.htm>.
10. Ike, F., Bourgade, F., Ohsawa, K., Sato, H., Morikawa, S., Saijo, M., Kurane, I., Takimoto, K., Yamada, Y.K., Jaubert, J., Berard, M., Nakata, H., Hiraiwa, N., Mekada, K., Takakura, A., Itoh, T., Obata, Y., Yoshiki, A., and Montagutelli, X. 2007. *Comp. Med.* 57: 272–281.
11. International Council for Laboratory Animal Science. 2008. History of ICLAS. [Online] <http://www.iclas.org/Document/History%20of%20ICLAS.pdf>.
12. International Council for Laboratory Animal Science. 2006. ICLAS Network for promotion of animal quality in research. [Online] <http://www.iclas.org/Document/ICLAS%20ANIMAL%20QUALITY%20NETWORK%20final.pdf>.
13. International Council for Laboratory Animal Science. 2007. Minutes of the ICLAS International Consortium Meeting. [Online] <http://www.iclas.org/Document/ICLAS%20International%20Consortium%20Meeting%20minutes%202007.pdf>.
14. International Council for Laboratory Animal Science. 2006. Performance Evaluation Program for Diagnostic Laboratories (PEP). [Online] <http://www.iclas.org/NetworkPEP.htm>.
15. Itoh, T. 1998. pp. 15–23. In: Proceedings of the 1998 US/ Japan Conference. National Academy Press, Washington, D.C.
16. Jacoby, R.O. and Lindsey, J.R. 1998. *ILAR J.* 39: 266–271.
17. Kagiya, N., Takakura, A., Terada, E., Sakurai, Y., and Suzuki, H. 1990. *Jikken Dobutsu* 39: 89–96.
18. Kagiya, N., Takakura, A., Terada, E., Sakurai, Y., and Suzuki, H. 1990. *Jikken Dobutsu* 39: 97–102.
19. Kraft, V., Meyer, B., Thunert, A., Deerberg, F., and Rehm, S. 1982. *Lab. Anim.* 16: 369–373.
20. Kunita, S., Chaya, M., Hagiwara, K., Ishida, T., Takakura, A., Sugimoto, T., Iseki, H., Fuke, K., Sugiyama, F., and Yagami, K. 2006. *Exp. Anim.* 55: 117–124.
21. Sakurai, Y., Suzuki, H., Kobayashi, N., Terada, E., Takakura, A., and Kagiya, N. 1990. *Jikken Dobutsu* 39: 549–555.
22. Scanziani, E., Gobbi, A., Crippa, L., Giusti, A.M., Giavazzi, R., Cavalletti, E., and Luini, M. 1997. *Lab. Anim.* 31: 206–211.
23. Scanziani, E., Gobbi, A., Crippa, L., Giusti, A.M., Pesenti, E., Cavalletti, E., and Luini, M. 1998. *Lab. Anim.* 32: 330–336.
24. Tagaya, M., Mori, I., Miyadai, T., Kimura, Y., Ito, H., and Nakakuki, K. 1995. *Lab. Anim. Sci.* 45: 233–238.
25. Wan, C.-H., Bauer, B., Pintel, D., and Riley, L. 2006. *Lab. Anim.* 40: 63–69.
26. Won, Y.S., Jeong, E.S., Park, H.J., Lee, C.H., Nam, K.H., Kim, H.C., Hyun, B.H., Lee, S.K., and Choi, Y.K. 2006. *Exp. Anim.* 55: 11–16.
27. Yagami, K., Goto, Y., Ishida, J., Ueno, Y., Kajiwar, N., and Sugiyama, F. 1995. *Lab. Anim. Sci.* 45: 326–328.