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Development and validation of ELISAs for monitoring bacterial and parasitic infections in laboratory rodents and rabbits

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Introduction

According to the FELASA recommendations for the health monitoring of rodent and rabbit breeding and experimental colonies *(Kraft et al. 1994; Rehbinder et al. 1996)* bacterial and parasitic infections are monitored by culture and microscopy respectively whereas viral infections are monitored by serology. The recommendation on the breeding colonies states that serological methods exist for the detection of antibodies to various bacterial pathogens e.g. *Bacillus piliformis* (now *Clostridium piliforme*), mycoplasmas and *Leptospira* spp. and suggests the possible use of serology for these infections as well as for *Treponema cuniculi*, *Encephalitozoon cuniculi* and *Toxoplasma gondii*.

Since 1985 we have explored the utility of serology, notably the enzyme-linked immunosorbent assay (ELISA), for the monitoring of animal colonies. Serology avoids the killing of animals and large numbers of animals can be more easily tested than by culture and results can be obtained within hours. Here we give a brief overview of our work and some experiences. Details can be found in a series of papers published between 1993 and 1999 in Laboratory Animals (LA) and the Journal of Experimental Animal Science (JEAnS) (Table 1). We started with non-X (hemin) and non-V (NADP) factor requiring *Pasteurellaceae* as such bacteria frequently occurred in all animal species. Later we extended our studies to growth factor requiring members of the family (*Haemophilus* sp) and bacterial species belonging to other families. The development and validation of the assays were done similarly for bacterial species or groups studied (Figure 1).

Serological relationship of bacterial strains

It would of course be convenient if e.g. all *Pasteurella pneumotropica* infections could be detected using a single antigen. Therefore we collected strains from different origin taken into consideration host species, disease status and geographical origin. For example our *P. pneumotropica* strains were isolated between 1948 and 1980 from healthy and diseased mice and rats from different continents. All strains were submitted to comprehensive morphological and biochemical studies and partly to cell wall lipid profiling (genetic methods were not used until recently), and representative strains were selected for serological studies.

To examine the serological relationship (cross-reactivity) of selected bacterial strains, merthiolate treated whole cell antigens (WCA) were prepared from all strains and immune sera were raised in SPF animals. The animal species used varied with the bacteria under study, the availability of uninfected animals and other considerations. Most antisera were prepared in rats or guinea pigs by primary subcutaneous immunization of WCA in adjuvant and boostering with plain WCA after 3 weeks. Initially we used Incomplete Freund's adjuvant, replaced it by Specol ® and at present immunize with plain WCA only as high antibody levels against bacterial antigens were also obtained without adjuvant. The use of adjuvant appeared, however, necessary to get good antibody levels to

Table 1. Serologic monitoring for bacterial and parasitic infections in rodents and rabbits

Microorganism	Assay	Mouse	Rat	G-pig	Rabbit	References
Actinobacillus taxon 5*	ELISA			1#		LA 1995, *29*, 59-65
Bordetella bronchiseptica	ELISA	1	1	1	1	LA 1993, *27*, 342-9; LA 1994, *28*, 335-9
Cilia associated respiratory bacillus	ELISA	2	2			Baltic J. LAS 1997, *7*, 51
Clostridium piliforme	IFA	1	1	1	1	
Corynebacterium kutscheri	ELISA	1	1	1		LA 1995, *29*, 294-9
Helicobacter bilis	ELISA	3	3			
Helicobacter hepaticus	ELISA	1	3			
Helicobacter muridarum	ELISA	3	3			
Helicobacter rodentium	ELISA	3	3			
Hemophilus sp H21 *	ELISA	1	1	1		JEAnS 1996/7, *38*, 147-152
Hemophilus sp H 35 *	ELISA	1	1			LA 1999, *33*, 91-4
Hemophilus sp H120 *	ELISA				1	LA 1999, *33*, 91-4
Mycoplasma pulmonis	ELISA	1	1			
Pasteurella multocida *	ELISA			1	1	
Pasteurella pneumotropica *	ELISA	1	1	1		LA 1994, *28*, 130-7;LA 1995, *29*, 307-13;. JEAnS 1994/5, *37*, 90-5
SP group *pasteurella* *	ELISA			1		LA 1995, *29*, 59-65
Streptobacillus moniliformis	ELISA	1	1	1		LA 1993, *27*, 350-7
Encephalitozoon cuniculi	IFA	2	2	1	1	LA 1988, *22*, 337-42, LA in press
Toxoplasma gondii	aggl.	2	2	1	1	

* members of the *Pasteurellaceae* family

1: FELASA primary list; 2: secondary list; 3: under study

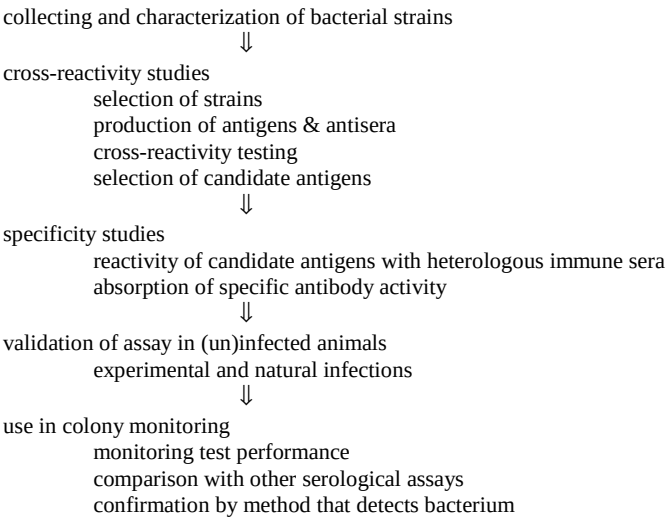
E. cuniculi. Animals were kept in isolators or micro-isolator cages to exclude intercurrent infection.

To test antibody activity, WCAs of Gram negative and positive bacteria were standardized on protein content of 7.5 and 15 µg / ml respectively and coated to high binding PVC microtiter plates. Every antiserum was tested with autologous antigen to determine optimal test conditions and then its optimal serum dilution was tested with homologous antigens. Finally, the antibody activity in homologous assays was calculated as percentage of the activity in the autologous assay and one or more candidate antigens were selected from the dataset. Mouse and rat strains of the recently discovered *Pathogenococcus inventoris* (Table 2) appeared serologically unrelated, but antigens from strains 3 and 4 might be able to detect infection of the bacterium in mouse and rat, respectively.

Selection of candidate antigen(s) for monitoring

From each cross-reactivity dataset one or more antigens that detected antibodies to as many homologous bacteria as possible were selected. In most studies it appeared that a single WCA of the group detected antibodies to all homologous strains used, so presumably would also be able to detect antibodies produced against strains occurring in most natural infections. Although this was also the case in *Corynebacterium kutscheri* here slight geographical antigenic differences among strains were apparent, suggesting that a European antigen might not necessarily detect all infections by for example Japanese strains of the bacterium. With *Bordetella bronchiseptica* it was

Fig. 1. Development and validation of serological assays



found that at least two antigens might be necessary to detect all infections in all animal species.

We have focused several studies on *Pasteurellaceae* from small laboratory animals. In this bacterial family, taxa occur that infect a single animal species only (this notably applies to guineapigs), but there are also less host specific taxa, for example *P. pneumotropica*). The taxonomic structure of the family is still under investigation and the serological relationship between the V - factor-requiring species (*Haemophilus*) and species that grow without this growth factor needs to be further studied. It can be envisaged that the panels of *Pasteurellaceae* antigens presently used for the monitoring of rodents and rabbits might have to be revised.

Candidate antigens should not detect antibodies to unrelated (heterologous) bacteria. The utility of the antigens was therefore further evaluated by testing antisera against heterologous pathogenic and commensal bacteria commonly occurring in SPF animals. The specificity of the assays was finally tested by absorption of antiserum against the candidate antigen with autologous and homologous antigens (which should decrease

Antibody activity) and with heterologous antigen (which should not). So far, our WCA seem to be specific, although occasionally limited reactivity with antiserum to one or another heterologous bacterium was observed, for *S. moniliformis* antigen showed some activity with antiserum to *Acholeplasma laidlawii*. Natural infection by such heterologous bacteria do, however, not necessarily lead to significant antibody levels, if infections occur at all.

Evaluation of assays in (un) infected animals

Preferably all animals that become infected by a pathogen rapidly develop measurable antibody levels. To get insight into the kinetics of antibody formation after infection and to compare the ELISA outcome with that of culture (‘golden standard'), we performed experimental infections in groups of animals. The protocol used was aimed to mimic natural infection as much as possible (Fig. 2). In each study live bacteria of the antigen - strain were dosed intranasally or orally to

uninfected SPF animals. Ten to 14 days post infection (p.i.) a limited number of animals of each group was killed and checked microbiologically to

Table 2. Cross-reactivity of *Pathogenococcus inventoris* strains from rodents by ELISA

Anti	collection		antiserum to strain						
gen	nr	host	1	2	3	4	5	.	n
1	CCUG 0001	mouse	**100#**	15	75	-	-		-
2	NIH 3456	mouse	90	**100**	**10**	-	-		20
3*	**LAM 64**	mouse	80	105	**100**	-	-		-
4*	**LAM 128**	rat	-	-	**100**	90	-		90
5	LAM 200	rat	20	-	80	**100**	**-**		**10**
..	....	..	..	..	..	..	**100**		
n	LAM 500	rat	10	-	-	30	10		**100**

autologous activity (in bold) considered 100 %; reactivity of antisera with homologous antigens calculated as percentage of autologous activity.

be sure the dosed animals would be a source of infection. Then uninfected animals were exposed to the deliberately infected counterparts and all animals were bled at periodic intervals. At the end of the study animals were bled, inspected for gross lesions and cultured for the bacterium. The Cilia associated respiratory (CAR) bacillus that cannot be cultured on artificial media was detected in tissue sections from the upper respiratory tract using a silver stain. *S. moniliformis* that appeared unculturable in experimentally infected rats, was detected in a repeat experiment by polymerase chain reaction (PCR). We did our studies under absolute containment within Gustafsson type isolators in order to exclude intercurrent infections and to protect animals in other facilities from the rodent pathogens studied. The outcome of our experimental infections and subsequent transmission studies suggest that antibodies in both groups can be measured 10 - 14 days after exposure to the bacterium.

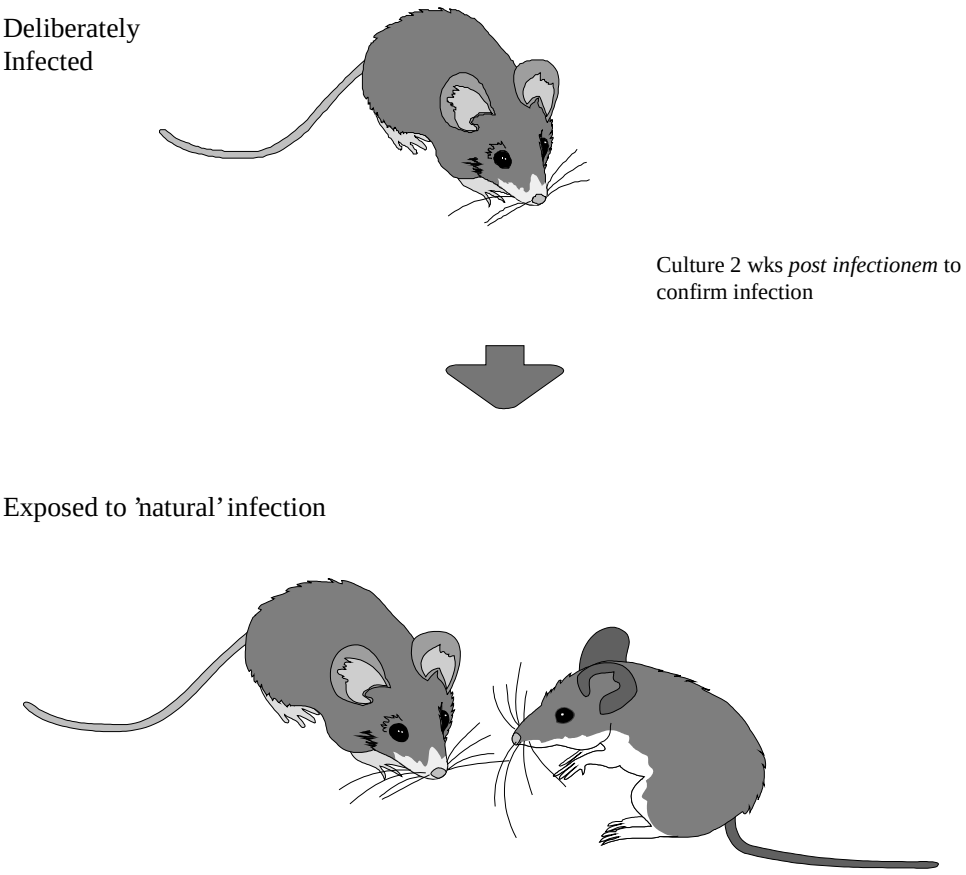
Comparisons of ELISA data with cultural results were also made in groups of animals from various colonies that were submitted to health status examination. One of very few 'discrepancies' was found in *P. pneumotropica* seropositive but culture negative LEW rats, in which infection by a serologically related *Haemophilus* sp was diagnosed after culture on chocolate agar. The validation of assays as described is considered an essential issue when testing laboratories would strive for accreditation *(Homberger et al 1999*).

Intra- and interlaboratory comparison

That 'results of serology for *B. piliformis* are currently controversial' *(Kraft et al 1996)* partly reflect different views on the interpretation of seromonitoring data for this bacterium. There are, however, more issues of concern. Recently we obtained *C. piliforme* positive rat sera from

another testing laboratory and confirmed the results. We returned some samples and our strong

Fig. 2. Set up of animal experiments for evaluation of antibody ELISAs



positive control serum to the laboratory of origin and surprisingly all were reported as negative. In order to monitor the performance of our ELISAs in every run, we include negative and positive control samples. In IFAs we test a positive control serum in a dilution series to evaluate the sensitivity of the run.

Whenever possible we compared our serological assays with analogue methods such as another in-house test. We thus compared ELISA and the previously used micro-agglutination (MA) test in *B. bronchiseptica* infection in rabbits and *P. pneumotropica* infection in rats and found a very good correlation. We also compared ELISA with IFA in *B. bronchiseptica* infection in guineapigs, *S. moniliformis* infection in rats, and *E. cuniculi* infection in rabbits. Such studies were done in collaboration with the few other laboratories that have explored serology for bacterial and parasitic infections in our field (former Zentralinstitut für Versuchstierzucht Hanover, Deutsches Krebsforschungszentrum Heidelberg, Institut für Labortierkunde Universität of Zurich, The Panum Institute University of Copenhagen).

Status quo and developments

Our studies have been focused on respiratory pathogenic bacteria (notably *Pasteurellaceae*) and some parasitic infections. Table 1 lists the assays that we have operational (indicated by 1 or 2). They comprise tests for microorganisms that are considered of primary importance to the different animal species (1 = FELASAs 'primary list') and additional microorganisms that should be monitored under certain circumstances (2 = 'secondary list'). Our present standard monitoring program for mice uses 9 antigens, whereas rat, guinea pig and rabbit sera are tested with 10, 11 and 6 antigens respectively. Recently we started the evaluation of ELISAs for some enteric pathogenic species such as *Citrobacter rodentium* and a number of *Helicobacter* sp. We invite other laboratories to participate in collaborative studies on these and other bacteria (Table 1, indicated by 3).

Although culture and serology often do not fully agree when data on individual animals are compared, both test types led, with rare exceptions, to the same conclusion for the animal colony: infected or not. Although serology often detects more infected animals than culture, it is difficult to reach a conclusion when very few samples (e.g. 1-2) are submitted for testing. Further the interpretation of ELISA data from tests using WCA from Gram-negative bacteria (including *Mycoplasma pulmonis*) may be complicated by age and animal strain related background activities. Furthermore, occasionally animals are encountered that react strongly to all antigens used. These phenomena are possibly related to poor colonization in the intestinal tract of rederived animals and can at best be detected when samples are tested for antibodies to all antigens. Positive serology is not proving infection. It is therefore advised to use bacterial culture or PCR for confirmation when important management decisions like culling an animal colony have to be taken.

We are fully aware of the possibility that animal colonies may remain seronegative when infected by pathogenic bacteria that do not cross react with the antigens used. We therefore encourage those submitting serum samples to report such events as the animal colony involved might be infected by bacterial strains from which new candidate antigens might be selected. All tests (about 16,000 annually) are operated under a quality system developed for laboratories in the health care sector which is based on EN 45001 criteria as is advocated by FELASA (*Homberger et al 1999).*

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