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A Multispecies Serological Microarray to Detect Exposure of Wildlife to Multiple Pathogens

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Abstract

Wildlife is a major reservoir for many human and livestock pathogens. Wildlife sampling is often very opportunistic and restricted. Many commercial immunoassays have not been licensed for routine diagnostic use in wildlife and standardized measurement of the antibody levels to different antigens has not been performed. As part of the EU-funded Wild Tech project and with the aim of providing the basis for a diagnostic test for use in surveillance studies, we evaluated the use of miniaturized protein A/G multiplex ELISA-based microarrayfor the detection of specific antibodies against multiple pathogens in single serum samples from wild mammal species.Sera obtained from wild cervids and wild boar were tested by array and conventional serodiagnostic assays. Using samples identified as positive or negative by conventional methods, we determined diagnostic cut-off points for cervids and wild boar sera tested by array for specific antibodies against 34 different bacterial and viral antigens. Of these 34, tests for *Mycobacterium bovis (TB), Brucella, Leptospira, Toxoplasma, Trichinella, Yersinia, Campylobacter* and Salmonella antigens produced comparable results with the standard serological tests. As a measure of agreement between array values and standard tests, Cohen kappa values for antigens tested with deer sera ranged from 0.41 to 0.92 and for wild boar sera from 0.43 to 1 (where 0 means no agreement and 1 means complete agreement). Despite the lack of success with some antigens, the promising results with others demonstrate proof-of-principle that this approach to rapid serodiagnosis could finally yield tests of great value for wildlife disease surveillance.

Introduction

Many pathogens are zoonotic and infect multiple animal species [1]. It has been estimated that approx. 75% of new and emerging diseases that have arisen in the last few decades are of wildlife origin [2,3]. Despite the fact that wildlife represents a major reservoir for many human and livestock pathogens, relatively little research has been conducted on the ecology of wildlife diseases, and for many diseases the wildlife reservoirs have not been identified [4]. Reasons for this include limited opportunities to gather samples, e.g. when hunting, need to immobilise the animals, autolysis of carcasses found in nature, difficult accessibility to the wild animals, and a lack of specific diagnostic tools for many wildlife species. Recognition of microbial antigens by the host immune system results in the production of specific antibodies, the detection of which is the basis for many well-established immunodiagnostic methods. Demonstration of specific antibodies in serum, plasma or meat juice has found applications in disease diagnosis [5-7] epidemiological studies and immune status assessment [8]. Many commercially available immunoassays employ purified antigens for antibody detection. However, whole-cell andcrude preparations have been used successfully to detect antibodies against various pathogens [5-10]. Although traditional tests undoubtedly have value, they do have drawbacks when screening for multiple pathogens, particularly where time, sample size and repeated sample availability are issues. More recent, array-based technologies [11-13] can overcome some of the major constraints

by allowing multiple targets to be investigated in a single process. Protein microarray technology has been used to perform quantitative and functional analysis of different proteins [14,15] and to detect exposure to pathogens [16-19]. Furthermore, the use of protein A and/or G conjugates allows assays to be used for numerous mammalian species. This is particularly useful for serological studies in wildlife where species-specific tools are frequently non-existent. Proteins A and G (and chimaeric A/G fusion proteins) are known to bind strongly to IgG of many mammalian species, and weakly to IgM and IgA in humans at least, and have been used in the development of diagnostic assays to detect exposure to pathogens in a range of animal species [20,21]. In addition to being cost-effective and labour-saving, testing multiple analytes is particularly important in wildlife surveillance, where samples are limited and precious. Although commercial serological tests for the detection of different pathogens in multiple hosts exist, none have been validated for routine diagnostic use in wildlife. As a proof-of-principle, we describe the development and testing of a multi-species multiplex protein array on the Array Strip platform (Alere Technologies GmbH, Jena, Germany) to detect antibodies against multiple pathogens in wildlife sera. The work was carried out as part of the Wild Tech project (www.wildtechproject.com), which is an international, multi-partner, EU-funded project (7th Framework Programme), which includes the aim to develop diagnostic tools to screen wildlife for a number of important pathogens.

Materials and Methods

Serum Samples

A total of 652serum samples were used in this study. All the samples were stored at −20°C until tested. The samples were divided into 3 groups:

Sera from cervids with known exposure status (n = 303): A total of 303 red deer (Cervus elaphus) sera collected in Spain during the hunting season were used for array validation. The infection status of samples for *Mycobacterium bovis* (TB), *Hepatitis E* (HEV), *Blue Tongue Virus* (BTV) and *Mycobacterium avium paratuberculosis* (pTB) had been previously determined using conventional serological methods [22-24].

Sera from wild boar with known exposure status (n = 225): A total of 225 Eurasian wild boar (*Sus scrofa*) sera collected in Spain during the hunting season were used for array validation. The infection status for *Mycobacterium bovi*s (TB) and *Brucella* spp had been previously determined using conventional serological methods [22,25].

Sera from deer with unknown exposure status (n=124): Sera from roe deer (*Capreolus capreolus*) (n=124) of unknown infection status collected in Sweden during the hunting season were tested. The animals were examined post mortem, with 43 of them showing clinical symptoms of diarrhoea.

Antigen selection/production

A range of native antigens (whole-cell preparations, protein fractions and LPS) and recombinant proteins from 18 pathogens were included in the array (see Table 1). Heat-inactivated whole-cell and crude antigen preparations were produced in-house. Purified recombinant antigens with reported serodiagnostic potential were purchased where commercially available. Recombinant Leptospira antigen LipL32 was kindly donated by Dr Medeiros, Fiocruz, Brazil. Prior to spotting, the antigens were dialysed overnight (Slide-a-Lyzer, Pierce) and buffers exchanged to PBS.

Array Platform

The ArrayStrip (AS) platform by Alere Technologies GmbH was chosen as the platform for test development (https://alere-technologies.com/products/lab-solutions/platforms/arraystrip-as.html). Two generations of arrays were used for preliminary development and assay optimisation (results not shown). For the third generation, dialysed antigens were spotted on the arrays

Table 1. Antigens included on the serology array. (LPS – lipopolysaccharide; w.c. – whole-cell; AE – acid-extract.)

Purified/recombinant	Supplier	Crude preps (in-house)
*Salmonella enteritidi*s LPS	Sigma	*Salmonella ser Enteritidis* w.c.
S. Typhimurium LP5	Sigma	*S.ser Typhimurium* w.c.
Francisella tularensis LPS	Reagensia AB	*Campylobacter coli* w.c. & AE
Brucella sp pbp26	fusionantibodies	*Campylobacter jejuni* w.c. & AE
Mycobact. bovis mpb70	Lionex	*Brucella abortus* 544 w.c.
*Mycobact. bovi*s mpb83	Lionex	*Brucella melitensis* 16Mw.c.
*Toxoplasma gondiire*c p29	Abcam	*Brucella suis* 1330w.c.
HepE virus HEV236	Prospec	*Brucella canis* R6/66w.c.
HepE virus HEV272	Prospec	*Brucella ovis* 63/290w.c.
*Hantavirus Puumala str Vranica**	Abcam	*F.tularensis ssp. holarctica* LVS4
*Hantavirus Dobrovastr Slovenia**	Abcam	*Coxiella burnetii* Lu 290
Aujeszky gE antigen	In-house - WBVR	*Escherichia coli* O157w.c.
Y. enterocolitica 03, 08 & 09 YOP ags	In-house –FLI	*Trichinella spiralis* 3rd instar larval ag
Leptospira spp LipL32	Dr Medeiros, Fiocruz, Brazil	*Toxoplasma* gondii
M.avium para-tb ags MAP 0210c, 1653 & 3527 [36]	Dr. Douwe Bakker, EU-project ParaTBTools	*Blue Tongue Virus* (BTV)

*Included on the array but no validation done due to a lack of samples with known serological status

Figure 1: Schematic of Wildtech Alere protein arrayversion 3 (1A) and stained array probed with Spanish wild boar serum, positive for TB (1B). Yellow squares represent metallic marks serving as orientation references for the processing software. Each array carries internal controls: spotting buffer (background control, square no. 146), recombinant protein A/G-HRP (conjugate reaction control, nos. 144, 145, - circled in 1B), and biotin-labelled protein (staining control, no. 147). Each antigen was spotted in triplicate. Spot 28 – TB antigen mpb70, 29 – TB mpb83. Stained spots are visualised and intensities quantified using the Alere Arraymate reader and IconoClust software.

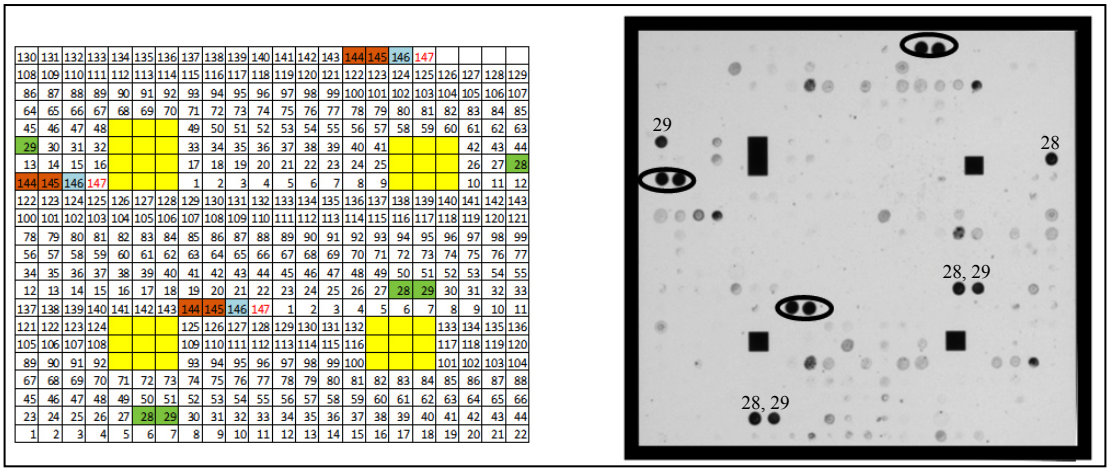
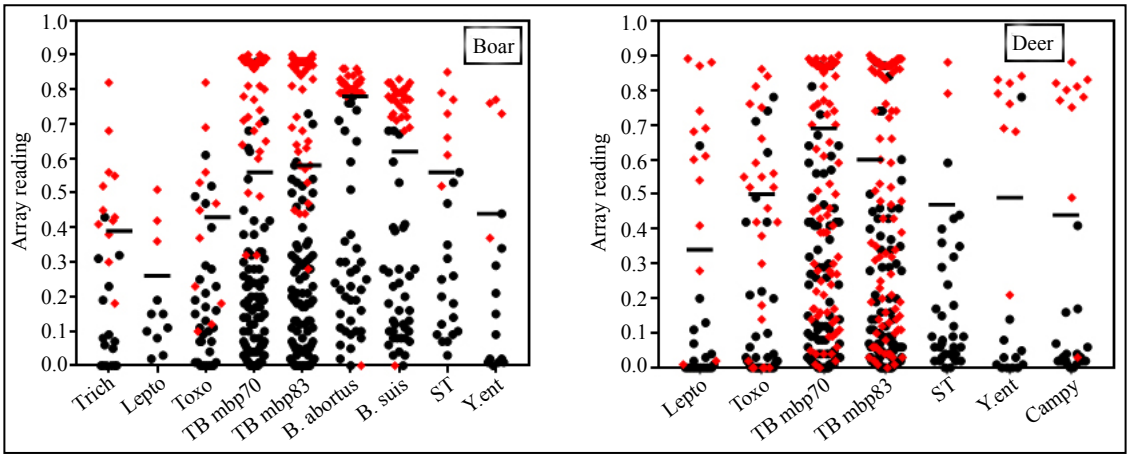


Figure 2. Array results for individual boar and deer sera with relation to confirmatory testing result. Red diamonds/ black circles = positive/negative by confirmatory testing (see Table 2). Black bars = array positive/negative cut-off values.



at a final concentration of 0.5µg/ml. The arrays are 4 mm x 4mm, allowing the deposition of up to 441 features, including reaction control and reference marker spots (Figure 1). Each AS consists of a standard 8-well strip with a high-quality microarray integrated in the bottom of each well. Up to 12 array strips can be inserted into a single micro plate frame. All antigens were spotted redundantly in triplicate and covalently coupled to the array glass surface. Recombinant protein-A/G-HRP was included as a positive control and buffer only as negative control. The detection system was based on the Protein Binding Kit (Alere Technologies) protocol. The hybridisation signals were visualised by an enzyme-catalysed precipitation reaction where catalytically induced precipitate directly correlates to the amount of target molecules binding to the array. Chimaeric Protein A/G HRP-conjugate (Thermo Fisher) was used to detect bound antibodies due to its broad specificity. After initial optimization studies (results not shown) Protein A/G-HRP was used at a dilution of 1:10000 for both deer and wild boar sera.

Test Protocol

Array Strips were first washed with 1% (w/v) dried, skimmed milk in P1 buffer (Alere Protein Kit, Alere) (150µl/well) for 5 min at 37°C shaking at 450rpm and then blocked with 3% skimmed milk powder in P1 buffer (100µl/well) for 5 min at 37°C shaking at 350 rpm. The liquid was removed, serum diluted 1/50 in 1% skimmed milk powder/P1 buffer was added to the wells (100µl/well), and the strips incubated for 30 min at 37°C shaking at 350 rpm. The sera were removed and the strips washed once as above. Protein A/G-HRP conjugate, diluted 1/10,000 in 1% skimmed milk powder/P1 buffer, was added to the wells (100µl/well) and the strips incubated for 30 min at 37°C shaking at 350 rpm. The conjugate was removed and the wells washed twice as above. The wash buffer was completely removed, D1 substrate (Alere Protein Kit) was added (10 µl/well), and the strips incubated for 10 min at 25°C without shaking. Finally, the substrate was completely removed and the arrays were immediately read on an Arraymate reader (Alere).

Measurements and Data Analysis

The Arraymate reader outputs were analysed by IconoClust software (Alere) according to the manufacturer's instructions .The signal intensity and local background were measured for each spot. Average extinction values of local background were subtracted from average extinction of the spot. For analysis,

a mean signal intensity value of all three replicate spots was used for each antigen type. The IconoClust software automatically calculates signal intensity values of the three spots carrying the same substance and the variation among them. Based on defined parameters for the current layout representing spot size, shape and coordinates, as well as background intensity and homogeneity, it checks the validity of values obtained for each substance. If predefined parameters are not achieved or an outlier of spot-to-spot variation for a certain substance is identified, the respective spot is deselected and eliminated from the calculation.

Corroborative Testing

Representative sera tested by array were also tested by established, non-array serological assays (Microscopic Agglutination Test [MAT], ELISA – Table 2) to corroborate array results and ascertain serological status so that cut-off values for the array antigens could be calculated. The MATs were performed by accredited diagnostic units (APHA and CVI). For the ELISAs, manufacturer's instructions were followed with the exception of the Salmonella and Yersinia ELISAs, which were modified by substitution of anti-porcine Ig-HRP with protein A/G-HRP in order to test deer sera.

Statistical Analyses

The cut-off values for each antigen and host species were determined as the mean value of the negative samples plus 2 standard deviations (SD) and then applied to the array results of positive and negative animals in order to determine sensitivities and specificities of each antigen. (Sensitivity = PA/P×100, where P= number of confirmed positives tested and PA the number of P positive by array, and N= total number of confirmed positives tested. Specificity = 100 – [(FP/N)×100], where N = total number of confirmed negative tested and FP = number of false positives, i.e. confirmed negatives with positive array values. To compare the performance of the array against established serological assays, kappa values with 95% Agresti intervals were calculated. Plots of mean versus difference, kappa values, binomial probabilities, sensitivity and specificity with Agresti confidence intervals were produced in Stata12 (Statacorp, College Station, Texas 77845 USA.

Results

Cut-off values determination and performance test

For the array development, 225 wild boar and 303 deer sera of known provenance were used to derive diagnostic cut-off points against a range of bacterial, viral and parasitic antigens spotted in triplicate on a miniaturised ELISA-based array. Signal intensity values ranged from 0.0 to 0.9. Reactive spots of positive sera were visually distinguishable from background and were identified using the IconoClust software, with signal intensity variations below 10 % between triplicate spots (Figure 1). Selected deer and wild boar sera deemed positive or negative to specified pathogens were tested by microarray and also examined using existing, standard serological assays and (Table 3).The array data of negative animals (as determined by the standard test) were then used to establish cut-off values for each antigen on the array. For some pathogens, more than one antigen was used for detection of specific antibodies. Microarray cut-off values were calculated as the mean signal intensity measured for negative animals plus 2 standard deviations. Cut-offs were then applied to the array results of positive and negative (see above) animals in order to determine sensitivities and specificities. Summaries of the determined cut-off values, as well as sensitivities and specificities for antigens tested on deer and wild boar sera are presented in (Tables 3, Table 4). There was a great degree of variation in the specificities and sensitivities of the antigens, with some showing considerable promise for serodiagnosis (e.g. *Trichinella* and TB). Some of the tested antigens had little merit for inclusion in the array due to lack of specificity, e.g. the *Aujeszky g* E antigen, where the mean value of negative animals was similar to that of positives and produced a cut-off (0.97) higher than any recorded array reading. Other antigens, including Hepatitis E, *Toxoplasma*

Table 2. Non-array assays used to determine serological status.(APHA -Animal and Plant Health Agency; WBVR – Wageninge Bioveterinary Research).

Pathogen	Test	Validated	Details
Brucella spp	ELISA	Pigs	COMPELISA 400 (APHA commercial kit)
Brucella spp	ELISA	Pigs	in-house rLPS cELISA
Leptospira	MAT	Pigs/Deer	APHA
Campylobacter	In-house ELISA	NV	APHA [37]
M. bovis (TB)	In-house ELISA	NV	[22]
M. paraTB	ELISA	Deer	LSI LSIVet Ruminant paraTB
Blue Tongue	ELISA	Deer	Ingezim BTV DR12.BTV.KO, Ingenasa, Spain
Hep E	In-house ELISA	NV	Peralta et al 2009
Q fever	ELISA	Deer	LSI LSIVet Ruminant Q fever
Toxoplasmosis	ELISA	Pigs/Deer	IDVet
Trichinella spp	ELISA	Pigs/Deer	Idexx
Salmonella	ELISA		Labor Diagnostik Salmotype pig screen
Yersinia spp	ELISA		Labor Diagnostik Pigtype Yopscreen
F. tularensis	MAT		WBVR

Table 3. Array test results for wild boar sera. Table shows mean signal intensity values of microarray for animals that were positive or negative by established serological methods. These results were then used to determine cut-offs for each antigen.

Antigen	Sera testing positive using established method			Sera testing negative using established method			cut-off*	Array	Array
	n	mean	SD	n	mean	SD	mean +2SD	sens*	spec*
Aujeszky (ADV)	4	0.61	0.12	21	0.54	0.22	0.97	0	ND
Trichinella 3rd instar	12	0.48	0.17	16	0.11	0.14	0.39	75	94
Toxoplasma gondii wc	11	0.4	0.29	25	0.18	0.26	0.71	18	92
T. gondii p29	11	0.41	0.24	25	0.13	0.15	0.43	55	84
Brucella abortus	31	0.79	0.15	38	0.31	0.24	0.78	94	100
Brucella suis	31	0.74	0.14	38	0.23	0.19	0.62	97	92
Lepto LipL32	3	0.43	0.07	8	0.1	0.08	0.26	100	100
Coxiella	0			7	0.27	0.21	0.69	ND*	ND*
M.bovis mpb70	76	0.83	0.12	80	0.18	0.19	0.56	92	94
M.bovis mpb83	76	0.81	0.14	80	0.2	0.19	0.58	87	97
Y.entero O3 YOP	4	0.12	0.03	12	0.03	0.04	0.1	50	92
Y.entero O8 YOP	4	0.29	0.14	12	0.07	0.1	0.26	75	100
Y.entero O9 YOP	4	0.66	0.19	12	0.13	0.15	0.44	75	100
S. Enteritidis LPS	7	0.47	0.29	17	0.07	0.07	0.21	86	94
S. Typhimurium LPS	7	0.61	0.19	17	0.15	0.14	0.43	86	94
S. Enteritidis wc	7	0.62	0.13	17	0.26	0.19	0.63	71	94
S. Typhimurium wc	7	0.71	0.11	17	0.23	0.17	0.56	86	100
HepE virus HEV236	10	0.01	0.01	13	0	0.01	0.02	0	ND*
HepE virus HEV272	10	0.03	0.04	13	0.02	0.02	0.06	0	ND*
M. para tbMAP3527	0	-	-	20	0.31	0.23	0.78	ND*	100
M. para tbMAP1653	0	-	-	20	0.1	0.09	0.28	ND*	95
M. para tb MAP0210c	0	-	-	20	0.25	0.22	0.7	ND*	95

n = number of tested sera, wc = whole-cell, AE = acid-extract. *ND – not determined due to lack of sera that tested positive or a complete lack of sensitivity. NB, n refers to the number positive for the pathogen/disease and not an individual antigen - e.g. there were seven Salmonella positive animals but the specific serovars were unknown.

whole-cell antigen, *Francisella*, paratuberculosis and BTV antigens, had very poor sensitivities (< 20%).Hanta virus antigens were included on the array, but their results could not be interpreted due to lack of samples of known serological status and no available method of determining the status of unknowns. There were also differences in the performance of the antigens between wild boar and deer sera, where results were generally more promising for the former.

Comparison of the array performance with a reference standard test

From testing the sera by array, applying cut-offs and calculating sensitivities and specificities, it was apparent that some antigens had more diagnostic value than others. In particular, the results for TB, *Brucella, Salmonella, Campylobacter, Yersinia, Toxoplasma, Trichinella and Leptospira* warranted further analyses. For these pathogens, the array performance was evaluated by comparison of the results with those ofestablished non-array serological assays (see Table 2) and calculation of the Cohen kappa values. Results for the established methods were classed as positive or negative, and the results for various antigens on the array were classed as positive or negative, according to pre-defined cut-off values. The array results for individual animals with relation to the confirmatory testing results are shown in (Figure 2). For some pathogens, the array test involved two or more antigens; if any of the antigens for a specific pathogen tested positive the result was classed as overall positive. Sensitivity and specificity values for deer and boar samples and calculated Cohen kappa values with 95% confidence intervals for some of the antigens are given in (Tables 5, Table 6). Kappa values close to 1 indicate near perfect agreement and close to 0 indicate no agreement (e.g. random). Cohen kappa values for antigens tested with deer sera ranged from 0.41 to 0.92 and for wild boar sera from 0.43 to1. To obtain a high confidence interval (CI), which indicates high certainty on the agreement between the tests, a large number of positive and negative samples are required.Where most samples were negative and few positive, the tests frequently agreed by chance, thus limiting the opportunity to assess discordance, as was the case with Salmonella and Brucella antigens tested with deer sera (Table 5). All values were significantly different from 0 at the 5% significance level, indicating better than random agreement of the array results with the reference standard serological assays.

Samples from roe deer with clinical symptoms

One hundred and twenty-four roe deer sera collected at hunting

Table 4. Array results for deer sera. Table shows mean array values for animals that were positive or negative by established serological methods. The mean and standard deviation from negative animals were then used to determine cut-offs for each antigen.

Antigen	Sera testing positive using established method			Sera testing negative using established method			Cut-off	Array	Array
	n	mean	SD	n	mean	SD	mean + 2SD	sens	spec
M. para tbMAP3527	3	0.11	0.1	17	0.13	0.23	0.59	0	82
M. para tbMAP1653	3	0.09	0.16	17	0.05	0.05	0.14	33	100
M. para tbMAP0210c	3	0.06	0.11	17	0.17	0.23	0.62	0	82
Toxoplasma gondii wc	8	0	0	19	0.04	0.09	0.23	0	ND*
T. gondii p29	8	0.27	0.3	19	0.1	0.2	0.5	50	86
Coxiella	2	0.53	0.12	18	0.37	0.14	0.65	0	ND
Lepto lipL32	13	0.56	0.3	22	0.06	0.14	0.34	77	95
M.bovis mpb70	89	0.5	0.33	94	0.21	0.24	0.69	46	95
M.bovis mpb83	89	0.46	0.34	94	0.17	0.22	0.6	44	94
Y.entero O3 YOP	12	0.16	0.18	24	0.03	0.04	0.11	42	92
Y.entero O8 YOP	12	0.45	0.21	24	0.08	0.13	0.34	83	96
Y.entero O9 YOP	12	0.69	0.2	24	0.11	0.19	0.49	83	96
S. Enteritidis LPS	2	0.42	0.55	33	0.09	0.12	0.33	50	94
S. Typhimurium LPS	2	0.69	0.13	33	0.24	0.26	0.75	50	100
S. Enteritidis wc	2	0.41	0.57	33	0.25	0.19	0.64	50	100
S.Typhimurium wc	2	0.84	0.06	33	0.15	0.16	0.47	100	97
Campy. coli AE	9	0.78	0.24	21	0.22	0.25	0.73	89	90
C. jejuni AE	9	0.78	0.12	21	0.09	0.18	0.44	100	95
F.tularensis w.c.	18	0.2	0.23	64	0.36	0.26	0.88	0	ND*
F.tular LPS	18	0.02	0.03	64	0.04	0.13	0.29	0	ND*
HepE virus HEV236	5	0	0.01	22	0	0.01	0.02	ND*	ND*
HepE virus HEV272	5	0.06	0.19	22	0	0.02	0.03	ND*	ND*
BTV4_RecVP2_Dom1	20	0.15	0.18	35	0.1	0.17	0.43	5	91
BTV4_RecVP2_Dom2	20	0.12	0.15	35	0.07	0.13	0.34	5	91
BTV4_RecVP2_Dom3	20	0.1	0.15	35	0.07	0.14	0.36	5	91
BTV4_RecVP5_Dom1	20	0.1	0.13	35	0.07	0.12	0.3	15	94
BTV4_RecVP7	20	0.06	0.09	35	0.05	0.09	0.22	10	91
Brucella abortus	0	-	-	26	0.08	0.12	0.33	ND*	100
Brucella suis	0	-	-	26	0.11	0.14	0.39	ND*	100

n = number of tested sera, wc = whole-cell, AE = acid-extract, ND – not determined due to lack of sera that tested positive or a complete lack of sensitivity. NB, n refers to the number positive for the pathogen/disease and not an individual antigen - eg there were 2 Salmonella positive animals but the specific serovars were unknown.

Table 5. The sensitivity, specificity and kappa values with their 95% confidence intervals of the array against reference established, non-array serological tests for deer sera. (*na – not determined due to lack of positive samples).

Pathogen	ELISA n -/+	Spec	Spec 95%CI	Sens	Sens 95%CI	kappa	k 95%CI
Salmonella	33/2	94	79-99	100	29-100	0.64	0.15-1.12
T. gondii	35/20	89	73-96	50	30-70	0.41	0.15-0.68
M. bovis (TB)	94/89	84	75-90	57	47-67	0.42	0.28-0.55
Leptospira	22/13	95	76-100	69	42-88	0.68	0.42-0.94
Brucella spp	26/0	96	80-100	na*	na?	na	na
Y. enterocolitica	8-Dec	92	62-100	88	51-100	0.79	0.52-1.07
Campylobacter	20/10	100	81-100	90	57-100	0.92	0.77-1.07

Table 6. The sensitivity, specificity and kappa values with their 95% confidence intervals of the array against reference established, non-array serological tests for boar sera.

Pathogen	Elisan -/+	Spec	Spec 95%CI	Sens	Sens 95%CI	kappa	k 95%CI
Salmonella	17/7	94	71-100	100	60-100	0.9	0.72-1.09
T. gondii	31/11	87	71-95	55	28-79	0.43	0.10-0.76
M. bovis (TB)	82/76	86	77-92	96	89-99	0.86	0.78-0.94
Leptospira	3-Aug	100	63-100	100	38-100	1	na
Brucella spp	38/31	92	78-98	97	82-100	0.88	0.77-0.99
Y. enterocolitica	4-Dec	100	72-100	75	29-97	0.82	0.47-1.16
T. spiralis	16/12	94	70-100	75	46-92	0.7	0.43-0.97

Table 7. Seropositivity (%), as detected by array, of Swedish deer with (n=43) and without (n=81) diarrhoeal symptoms.

Pathogen	Diarrhoea (%)	No diarrhoea (%)
Salmonella	2.3	0.0
Campylobacter spp	72.1	25.9
Brucella spp	0.0	0.0
Y. enterocolitica	18.6	11.1
Leptospira spp	2.3	0.0
M. bovis (TB)	9.3	16.0
T.spiralis	0.0	0.0
T.gondii	11.6	14.8

in Sweden were tested on the array. Forty-three were from deer examined by necropsy and showed signs of diarrhoea, and 81 were from apparently healthy animals. A summary of the array results of the 124 animals is given in (Table 7). The array results revealed elevated levels of antibodies to a number of enteric pathogens in the deer with diarrhoeal symptoms compared to the healthy animals. Interestingly, the majority (72%) of the samples associated with the presence of clinical symptoms of diarrhoea were seropositive for Campylobacter antibodies compared to 25.9% in the asymptomatic animals ($p<0.0001$, Fisher's exact test). There were also higher numbers of *Yersinia* seropositive animals in the diseased group (18.6% vs. 11.1%) although this was not significant ($p=0.28$). Antibodies that indicate exposure to *Toxoplasma gondii*, TB, *Leptospira and Salmonella* were also detected in both groups.

Discussion

Since their development in the early 1970s, ELISAs have remained in wide use in their original format. Besides, there are now also expanded formats in use with modifications that allow testing multiple analytes at the same time without compromising individual readouts. Microarray technology hasrevolutionisedmany aspects of biological research, allowing for the study of many thousands of gene transcripts or protein-protein interactions to be monitored simultaneously reviewed in [26,27]. Here we describe the development of a microarray based on multiplexed ELISAs that fulfils the criteria of low complexity, high through-put potential and lower demands on experimental time and manpower yet with good analytical performance. Despite wildlife representing major reservoirs of many important pathogens [2], very few commercially available tests have been developed/adapted and validated for their serodiagnosis. There are studies that describe use of individual ELISA-based assays e.g. [28,29], however, to our knowledge, this is the first study that describes evaluation of a multiplex protein array for detection of exposure to multiple pathogens in different wildlife species. Since biological samples of wildlife are frequently precious and hard to come by, testing for multiple targets in a single test would be particularly useful for wildlife surveillance programmes.Furthermore, use of a protein A/G conjugate allows the single serology array to be used for simultaneous testing of multiple mammalian species, including wild boar and deer as reported here. Using our microarray, antibody responses to multiple, diverse antigens can be measured in each well of a 96-well plateusing the same amount of time it takes to complete one conventional ELISA. Importantly, a total of only 2µl of sera are needed, which would only suffice for a single conventional ELISA test. We used a combination of crude and purified antigens, including heat-inactivated bacterial cells, recombinant proteins and LPS printed onto3d epoiyde based structure-coated glass slides to develop a high-throughput microarray capable of simultaneously detect ingantibodies to multiple pathogens. The array was used to test 652sera from wild boar and deer. The use of whole-cell and crude antigens in immunoassays avoids the need for expensive or time-consuming purification and offers the advantage of presenting surface antigens in their native form, thereby simulating antigen–antibody reactions in vivo [6]. Before selection for the array, performances of the antigens were tested in individual in-house ELISAs, using experimentally-raised positive and negative control antisera (results not shown). Sensitivities and specificities of the antigens on the array were determined by testing the deer and wild boar sera with conventional ELISAs and MATs to identify sero-positives and negatives for the pathogens of interest. The aim of this work was to demonstrate proof-of-principle

in establishing a robust, multiplex antigen array as a tool for wildlife health surveillance and to compare its coherence to established serological tests. The work was hindered by a lack of appropriate control sera. Ideally, control sera would come from animals of defined bacteriological and immunological status (naïve/infected/previously infected) but this was not always possible for our work as appropriate samples from the target species were not available. However, established serological tests were available for many of the chosen pathogens, although not always validated for boar or deer sera, so the results of these were used for comparison with array results. Cohen kappa values confirmed that the array results for a number of pathogens (*Salmonella, Yersinia, bovine TB, Brucella, Trichinella, Toxoplasma, Campylobacter and Leptospira*) were consistent with those of the established serological tests. We determined cut-off values for the array by the recognised but relatively simplistic method of using mean plus two standard deviations of values from uninfected animals [30]. For 'proof-of-principle' this approach was deemed good enough, but further work using larger populations covering a full range of immunological status would include a more rigorous approach to achieve greater test accuracy [31]. Once cut-offs were established, we then determined the sensitivities and specificities of the antigens on the array. These are key criteria when considering the usefulness of a test. Clearly, 100% sensitivity and specificity would be the ideal but this is rarely the case even with the most accurate test. The intended purpose of the test is a key consideration when assessing sensitivity/specificity. For diagnostic tests both criteria need to be as high as possible as there could be serious, direct consequences for mis-diagnosis, such as failure to treat a patient assumed to be healthy or slaughter of an animal assumed to be infected. Screening tests are usually easier and cheaper to perform but are more imprecise [32]. Screening tests can also be used for different reasons which may affect the test criteria. For example, if a test is used to screen individuals for disease it is important not to miss any positives (ie high sensitivity) whereas specificity may be less important as false positives can be determined by further testing. Screening tests are also used to study populations for epidemiological and risk assessment purposes, where valuable data can still be obtained from tests with relatively low sensitivities and specificities. Raising or lowering the cut-off will affect the performance of the test. Thus, it is possible that even a relatively poor test can yield valuable population data by having multiple cut-off points which define low/moderate/high risk categories for the population to be/become infected [30]. On our array there was a wide range of specificities and sensitivities but there were enough with levels sufficient to validate the usefulness of the approach for screening purposes and warrant further research. Interestingly, the array results suggest 17 of 124 roe deer from Sweden were serologically positive for TB. Sweden was declared free of bovine TB in 1958 and wildlife in Sweden is also considered free of bovine TB [33]. A systematic wildlife disease surveillance programme has been in place in Sweden since the '40´s. No cases of bovine TB have been detected in cervids, badgers (*Meles meles*) or any other wildlife species investigated, except for 2 historical cases in free-ranging moose (*Alces alces*), one in 1940 [34] and one in 1943 [35]. However, bovine TB was detected in several herds of farmed fallow deer (*Dama dama*) in 1991; the source was identified as a consignment of imported deer [36]. Successful eradication measures were applied, including extensive trace back investigations, stamping out and a control programme. Surveillance of farmed deer, including meat inspection, tuberculin testing, necropsy and culture has not identified any further cases indicating that most probably bovine TB was eradicated from the farmed deer in Sweden [33]. Nonetheless, absence of bovine TB infection at the individual animal level is very difficult to confirm due to limitations in test sensitivity. Therefore, even though the epidemiological TB status of animals in Swedish indicates a minimal probability of the roe deer to have been infected, true infection cannot be completely excluded. Possibly, lack of specificity of our assay may explain the positive serology. Other members of the M. *tuberculosis* complex may express high levels of MPB70/83, for example *Mycobacterium microti* [37]. Thus, our findings warrant further investigation to assess specificity to TB. For a number of pathogens (e.g. Aujeszky's Disease and F. *tularensis*) array results appeared to be non-specific or insensitive and showed little or no correlation with established tests. This lack of correlation may be due to the use of inappropriate antigens that either lacked specificity or, possibly, were unable to bind properly during array production. Further work should enable improved antigen selection and/or improved array printing to enable the development of more comprehensive arrays. Thus, our work has demonstrated that a rapid and technically simple test for the serodiagnosis of multiple pathogens in multiple animal species yielding results consistent with established serological tests is a viable prospect. Furthermore, the format has the capability to include many more antigens, making it even more useful and cost-effective. In addition, the use of different HRP conjugates (e.g. anti-avian Ig or anti-isotype specific) would also increase the usefulness of this approach and warrants further research and development.

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