Chapter 9
An Ante-Mortem Test for Bovine Spongiform Encephalopathy Involving "Myelin-Acinetobacter-Neurofilaments" (MAN) Tested in 12 Strains of *Acinetobacter* Bacteria

9.1 Introduction: Bovine Spongiform Encephalopathy and Environmental Bacteria

Previous studies have shown that animals afflicted by bovine spongiform encephalopathy (BSE) have elevated levels of specific antibodies to *Acinetobacter* bacteria but not to six other common environmental microbes.

Furthermore such animals have specific autoantibodies to bovine myelin and to bovine neurofilaments.

The possibility arises that such observations could be used to develop an ante-mortem test for the presence of bovine spongiform encephalopathy in the early stages of the disease.

When the bovine spongiform encephalopathy was first described in the 1980's, it was feared that consumption of meat from such animals might be transmitted to humans and cause a neurological disorder resembling Creutzfeldt-Jakob disease.

Acinetobacter bacteria are found usually in the biological environment of man and animals. These bacteria can be isolated from soil, sewage and also from muddy water. In the case of the feeds provided to the BSE animals, contamination with *Acinetobacter* bacteria may have occurred during collection of animal brain and other offal material from abattoirs where muddy waters are frequently encountered. This was probably the most likely way that *Acinetobacter* components were incorporated in "meat-and-bonemeal" (MBM) preparations.

Clearly MBM supplements were involved since the statutory ban on their use has led to a significant decrease in BSE in the UK, although not to zero levels.

Early detection of the disease was considered of paramount importance in providing safe meat for human consumption.

An attempt to develop an ante-mortem test for bovine spongiform encephalopathy was initially approved and sponsored by the Ministry of Agriculture, Fisheries and Food (MAFF) and subsequently endorsed by the newly baptised Department of the Environment, Food and Rural Affairs (DEFRA).

A. Ebringer, *Multiple Sclerosis, Mad Cow Disease and Acinetobacter*,
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Table 9.1 Source and reference number of bacterial cultures

Source of bacterial culture	Name	Species number	Catalogue number
Public health laboratory	*Acinetobacter calcoaceticus*	sp1	ATCC 23055
	Acinetobacter baumannii	sp2	ATCC 19606
	Acinetobacter	sp3	ATCC 19004
	Acinetobacter haemolyticus	sp4	ATCC 17906
	Acinetobacter junii	sp5	ATCC 17908
	Acinetobacter	sp6	ATCC 17909
	Acinetobacter johnsonii	sp7	ATCC 17909
	Acinetobacter lwoffii	sp8	NTCC 5866
	Acinetobacter	sp9	ATCC 9957
	Acinetobacter	sp10	ATCC 14924
	Acinetobacter radioresistens	sp12	
	Acinetobacter	sp16	ATCC 17988
	Acinetobacter	sp17	

Wilson et al. (2003)
NCTC National Collection of Type Cultures, *ATCC* American Type Culture Collection

9.2 Sera from Animals with and Without BSE, Test Bacteria and Peptides

This study involved a much larger number of sera from 189 BSE positive animals and compared to 127 BSE negative cows and 64 healthy control cows.

The study was carried out on sera obtained from 380 cows, each test was done blind and in duplicate, with the 3 different isotypes (IgA, IgG and IgM) and 4 separate peptides, involving altogether 9,672 separate estimations.

Acinetobacter bacterial cultures were provided by Dr. Kevin Towner from the Public Health Laboratory, Nottingham, UK (Table 9.1).

Peptides and ELISA studies were carried out as previously described.

The first test 15-mer peptide was "RGSLS(RFSWGAE)GQK", which represents amino acid residues 107–121 of bovine myelin basic protein (MBP).

The second brain 15-mer peptide studied was "KQLQ(ELEDK)QNADIS" which refers to amino acid residues 331–345 of bovine neurofilament.

This peptide shows molecular mimicry with the bacterial sequence "RALI(ALEDK)SNFIEA" which represents amino acid residues 208–222 of protocatechuate 3,4 dioxygenase of *Acinetobacter calcoaceticus*.

Further analysis showed a sequence identity involving bovine prion (RPVDQ) (arginine-proline-valine-aspartic acid-glutamine) spanning residues 175–179 which was also present in uridine-di-phosphate-N-acetyl-glucosamine-l-carboxy-vinyl transferase (RPVDQ) spanning residues 121–125 of *Acinetobacter calcoaceticus*.

The question arose whether BSE affected animals had produced antibodies against these crossreacting sequences found in bovine prions and also in *Acinetobacter* bacteria.

Further peptides were prepared by the methods previously described. The 14-mer test peptides used were QVYY(RPVDQ)YSNQN, which represents amino acid residues 171–184 of bovine prion and AIGS(RPVDQ)HLKAL, which represents amino acid residues 117–130 of UDP-N-acetyl-glucosamine-l-carboxy-vinyl transferase, a molecule present in *Acinetobacter calcoaceticus*.

9.3 Antibodies to *Acinetobacter* Peptides and Autoantibodies to Corresponding Bovine Brain Peptides

Significant levels of antibodies to the 3 different isotypes (IgA, IgG and IgM) were found against both the *Acinetobacter* peptides and bovine brain peptides involving altogether 24 separate comparisons.

The t-values were calculated, all of which were significant at a p-value of $p<0.001$ (Table 9.2).

Significantly elevated levels of IgA autoantibodies against the bovine prion peptide were found in 189 BSE positive animals when compared to 127 BSE negative animals ($t=10.44$, $p<0.001$) and 87 healthy controls ($t=9.94$, $p<0.001$).

Furthermore significantly elevated levels of IgA antibodies against the corresponding *Acinetobacter* peptide were found in the BSE positive animals when compared to BSE negative animals ($t=13.20$, $p<0.001$) or healthy animals ($t=13.61$, $p<0.001$).

These results would seem to indicate that prions, at least over the sequences studied, can be the subject of immune activity. Therefore the suggestion that prions are not involved in immune responses would appear to require revision.

9.4 An Ante-Mortem Test for BSE

In this study an attempt was made to develop an ante-mortem test for BSE using ELISA.

The ELISA study was carried out with 12 different species of *Acinetobacter,* as well as bovine brain peptides, in each case involving 28 BSE positive and 18 BSE negative animals, in an attempt to determine which bacteria gave optimal results in a M.A.N. (Myelin-*Acinetobacter*-Neurofilament) assay.

The 12 *Acinetobacter* strains were provided by Dr. Kevin Towner from the Public Health Laboratory in Nottingham (Table 9.1).

To improve the reliability of the assay, short synthetic peptide sequences of bovine brain molecules showing molecular mimicry to *Acinetobacter* bacteria were used, rather than total extracts of bovine myelin basic protein or bovine neurofilaments, in an endeavour to reduce non-specific antibody bindings. Total immunoglobulin (Ig) levels to myelin, *Acinetobacter* and neurofilaments were used in the M.A.N. assay.

Table 9.2 Statistical results (t-values and significance) of anti-peptide antibody levels in the sera of 189 BSE positive, 127 BSE negative and 87 healthy control cows

	IgA	IgG	IgM						
	BSE+ve vs controls	BSE+ vc vs BSE -vc	BSE -vc vs controls	BSE+ vc vs controls	BSE+ vc vs BSE -vc	BSE -vc vs controls	BSE+ vc vs controls	BSE+ vc vs BSE -vc	BSE -vc vs controls
Acinetobacter-myelin	16.06	15.89	NS	15.41	15.85	NS	11.16	9.92	2.33
	<.001	<.001		<.001	<.001		<.001	<.001	<.005
Acinetobacter-neurofilament	16.07	16.27	NS	13.75	14.51	NS	6.72	8.62	NS
	<.001	<.001		<.001	<.001		<.001	<.001	
Bovine-myclin	18.11	17.58	NS	17.55	17.87	NS	16.38	15.23	NS
	<.001	<.001		<.001	<.001		<.001	<.001	
Bovinc-neurofilament	16.56	17.20	NS	15.62	13.32	NS	5.72	6.48	NS
	<.001	<.001		<.001	<.001		<.001	<.001	

NS not significant

The 15-mer test peptides were prepared as previously described and involved "RGSLSRFSWGAEGKQ" which represents amino acid residues 107–121 of bovine myelin basic protein (MBP) and "KQLQELEDKQNADIS" spanning amino acid residues 331–345 of bovine neurofilaments.

The M.A.N. (Myelin-*Acinetobacter*-Neurofilament) index was calculated in both BSE positive and BSE negative cows as follows:

$$\text{M.A.N. Index} = (\text{Ig MBP} \times 10) \times (\text{Ig Acinetobacter} \times 10) \times (\text{Ig Neurofilament} \times 10)$$

The 99.9 % confidence limits (CL) of the controls were calculated as follows: = Mean ± 3 SD (standard deviations)

The sensitivity and specificity were determined by the method of Anderson (1976).

$$\text{Sensitivity} = \frac{\text{Number of BSE positive animals above 99.9\% CL}}{\text{Total number of BSE positive animals}} \times 100(\%)$$

$$\text{Specificity} = \frac{\text{Number of BSE negative animals below 99.9\%CL}}{\text{Total number of BSE negative animals}} \times 100(\%)$$

9.5 Sensitivity and Specificity of Ante-Mortem Test

Total antibody (IgA, IgG and IgM) measured against the 12 different strains of *Acinetobacter* in the BSE positive animals showed that all had significantly elevated levels ($p < 0.001$) when compared to the 18 controls, the highest difference being given by *Acinetobacter johnsonii* (Table 9.3).

Table 9.3 Antibody responses (mean ± SE) to different strains of *Acinetobacter*

Name	BSE positive	Controls	t-value	Statistical significance
A. calcoaceticus (spl)	0.668 ± 0.031	0.298 + 0.098	8.66	$p < 0.001$
A. baumannii (sp2)	0.452 ± 0.013	0.251 ± 0.030	7.02	$p < 0.001$
Acinetobacter (sp3)	0.402 + 0.011	0.230 + 0.015	9.27	$p < 0.001$
A. haemolyticus (sp4)	0.376 ± 0.012	0.237 + 0.013	7.79	$p < 0.001$
A. junii (sp5)	0.245 ± 0.011	0.145 ± 0.011	5.95	$p < 0.001$
Acinetobacter (sp6)	0.399 ± 0.016	0.222 ± 0.021	6.74	$p < 0.001$
A. johnsonii (sp7)	0.627 ± 0.014	0.340 + 0.014	13.52	$p < 0.001$
A. lwoffii (sp8)	0.494 + 0.024	0.228 + 0.016	8.07	$p < 0.001$
Acinetobacter (sp9)	0.506 ± 0.016	0.268 + 0.023	8.63	$p < 0.001$
Acinetobacter (sp10)	0.383 + 0.010	0.266 + 0.017	6.34	$p < 0.001$
Acinetobacter (sp16)	0.425 ± 0.015	0.254IO.022	6.65	$p < 0.001$
Acinetobacter (sp17)	0.415 + 0.020	0.223 ± 0.026	5.94	$p < 0.001$

Wilson et al. (2003)

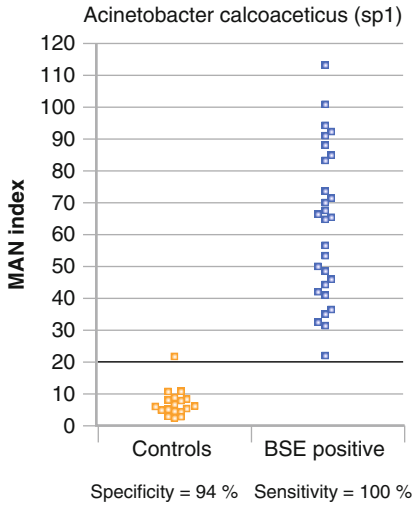

Fig. 9.1 Sensitivity and specificity of *Acinetobacter calcoaceticus (sp 1)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

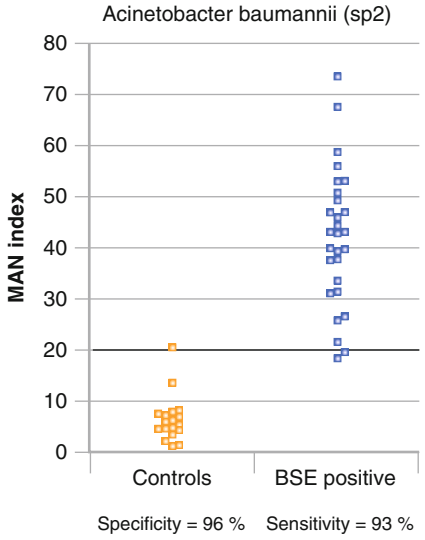

Fig. 9.2 Sensitivity and specificity of *Acinetobacter baumannii (sp2)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

Furthermore, significantly elevated levels of antibodies to bovine myelin basic protein peptides ($t = 6.93$, $p < 0.001$) and neurofilament peptides ($t = 10.09$, $p < 0.001$) were present in the BSE affected animals when compared to the 18 controls.

The sensitivity and specificity of the M.A.N. assay was calculated for each of the 12 *Acinetobacter* strains and summarized in the following figures (Figs. 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 9.10, 9.11 and 9.12).

Five out of these 12 species had a sensitivity of 100 % and specificity of 100 % in detecting BSE affected animals.

These were *Acinetobacter* (sp.3), *Acinetobacter haemolyticus, Acinetobacter* (sp.9), *Acinetobacter lwoffii* and *Acinetobacter johnsonii* (sp.7).

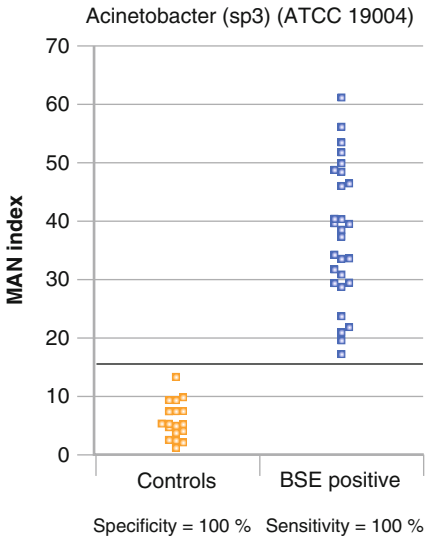

Fig. 9.3 Sensitivity and specificity of *Acinetobacter (sp 3) (ATCC 19004)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

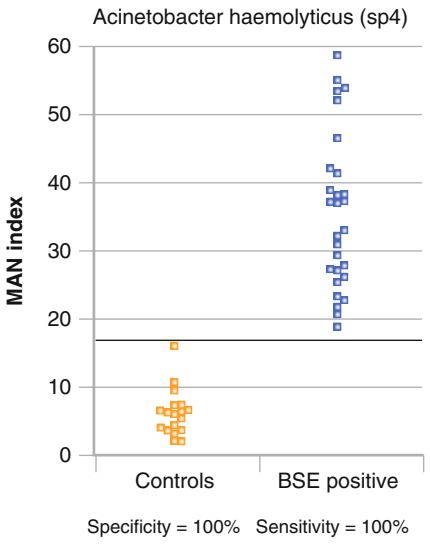

Fig. 9.4 Sensitivity and specificity of *Acinetobacter haemolyticus (sp 4)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

The remaining seven *Acinetobacter* strains also gave high readings close to 100 % in sensitivity and specificity (Wilson et al. 2003).

Acinetobacter calcoaceticus can survive on dry surfaces for up to 60 h (Musa et al. 1990).

Acinetobacter (sp.3) is found in soil and clinical specimens.

Acinetobacter baumannii is the commonest clinical isolate, especially from tracheal aspirates and wound swabs (Towner 1996).

Acinetobacter haemolyticus (sp.4) has been isolated occasionally from patients, the hospital environment and activated sludge samples.

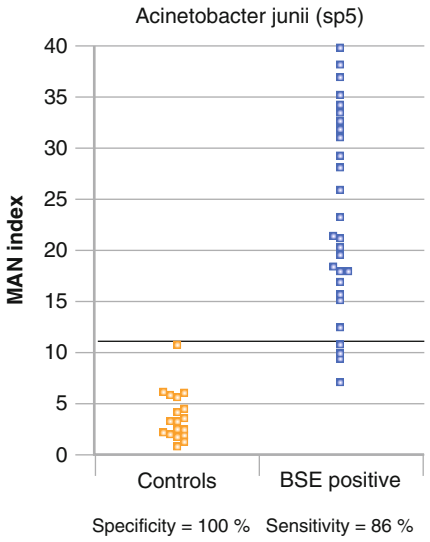

Fig. 9.5 Sensitivity and specificity of *Acinetobacter junii (sp 5)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

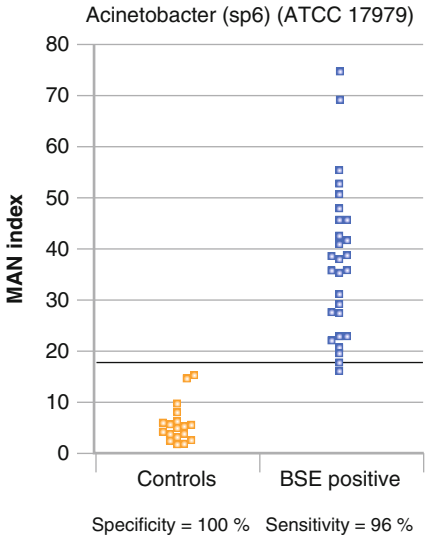

Fig. 9.6 Sensitivity and specificity of *Acinetobacter (sp 6) (ATCC 17979)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

Acinetobacter johnsonii (sp.7) and *Acinetobacter lwoffii* (sp.8) have been isolated from animals and animal products, soil and activated sludge samples but rarely from human clinical specimens.

However *Acinetobacter johnsonii* (sp.7) and *Acinetobacter lwoffii* (sp.8) can be isolated from various food sources, including fresh and spoiled meat, fish, vegetables, raw milk and cheese (Gennari and Lombardi 1993).

The ecological niche of *Acinetobacter* (sp.9) has not been clearly defined.

It is interesting to note that most of these *Acinetobacter* strains are found in soil and sludge materials. Whether the MBM feed preparations had been contaminated by such materials during collections from abattoirs is at the moment unknown.

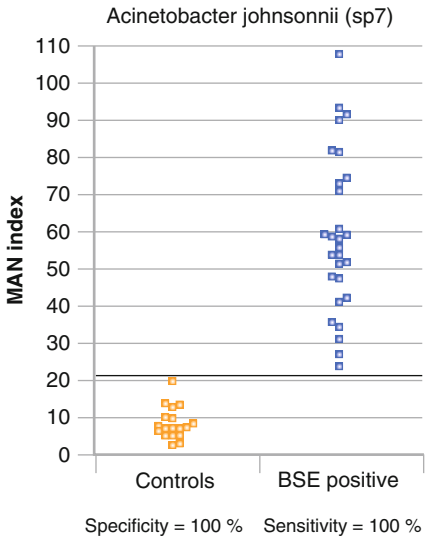

Fig. 9.7 Sensitivity and specificity of *Acinetobacter johnsonii (sp7)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

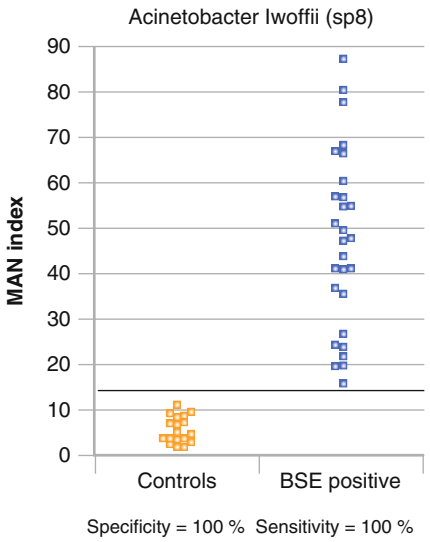

Fig. 9.8 Sensitivity and specificity of *Acinetobacter lwoffii (sp 8)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

9.6 Discussion and Conclusions

The M.A.N. assay has been used in this study to compare the ability of 12 different species of *Acinetobacter* to detect BSE affected cattle.

Short synthetic peptide sequences of bovine brain molecules, showing molecular mimicry to *Acinetobacter* bacteria have been used, rather than total extracts of bovine myelin basic protein or bovine neurofilaments in an attempt to improve the sensitivity and accuracy of the test assay.

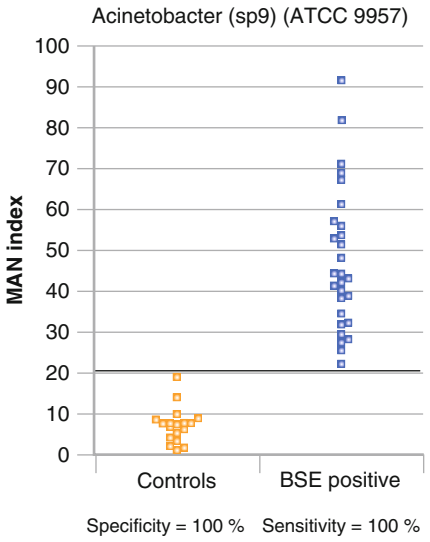

Fig. 9.9 Sensitivity and specificity of *Acinetobacter (sp9) (ATCC 9957)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

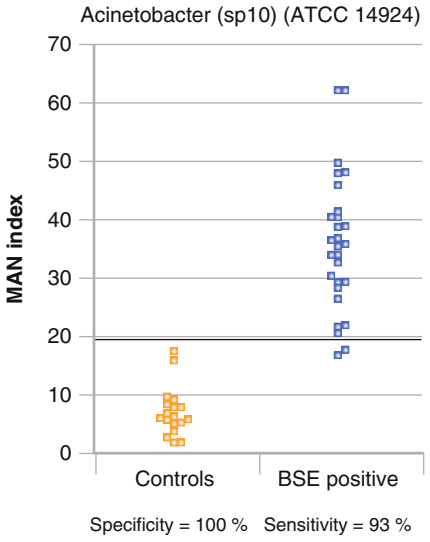

Fig. 9.10 Sensitivity and specificity of *Acinetobacter (sp10) (ATCC 14924)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

Five of the twelve *Acinetobacter* species studied had a sensitivity of 100 % and a sensitivity of 100 % in detecting BSE affected cattle and the remaining seven gave also high readings close to 100 %.

The important question arises how damage to brain tissues occurs with *Acinetobacter* or even *Pseudomonas* infection. Clearly antibody cytotoxic activity occurs with complement activation and the onset of inflammatory cascades leading to production of neurological damage.

The measurement of anti-*Acinetobacter* antibodies could be used to identify animals affected by BSE so that they could be excluded from the abattoirs and entry into the human food chain.

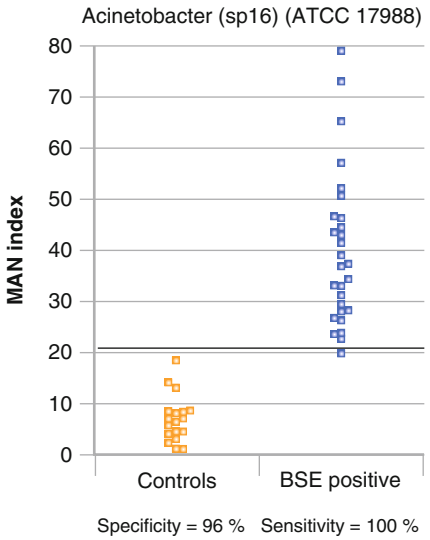

Fig. 9.11 Sensitivity and specificity of *Acinetobacter (sp16)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003))

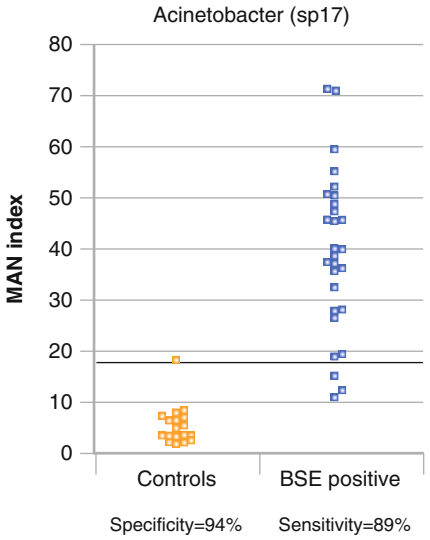

Fig. 9.12 Sensitivity and specificity of *Acinetobacter (sp 17)* in detecting BSE affected animals using a M.A.N. assay (Data from Wilson et al. (2003)

More extensive investigations are required to determine the value of measuring anti-*Acinetobacter* antibodies in dairy herds as well as in animals used for meat production.

The M.A.N. assay appears to provide reliable results but more field studies are clearly necessary to determine its value for general veterinary services.

The pathology of BSE and its onset in animals remains unresolved but at least the M.A.N. assay provides a new way of identifying animals that could be carrying the disease. Suspect or compromised animals need to be excluded from human consumption and this test could assist in early identification.

It is to be noted that the test can be carried out on 1 ml of blood.

References

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Wilson C, Hughes LE, Rashid R, Ebringer A, Bansal S. Antibodies to *Acinetobacter* bacteria and bovine brain peptides, measured in bovine spongiform encephalopathy (BSE) in an attempt to develop an ante-mortem test. J Clin Lab Immunol. 2003;52:23–40.