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BOVINE THEILERIOSIS – DISTRIBUTION AND SIGNIFICANCE OF MAJOR PIROPLASM SURFACE PROTEIN (MPSP) TYPES

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Abstract

Numerous outbreaks of bovine theileriosis have occurred in eastern Australia since 2007. DNA extracts taken from blood of the initial clinical cases in NSW, when tested in Japan were found to contain three types of organisms that belonged to the group of protozoa referred to as “benign theilerias”. The types are defined by specific DNA sequences within the gene that encodes the major piroplasm surface protein (MPSP) of these organisms. Of the three MPSP types, one (Buffeli) has been present in Australia for many years without associated disease signs. A second (Chitose) was identified in normal cattle exported to Japan many years ago. From the initial clinical cases, these two types in addition to a third type, associated with outbreaks of theileriosis overseas (Ikeda), were found. Our study sought to identify the distribution of MPSP types here, by survey of cattle herds in the three eastern mainland states. Secondly, individual cattle in herds considered at risk of theileriosis were monitored to determine the presence of MPSP types over time. This information is required to determine basic epidemiological data on theileriosis in Australia, and help identify higher risk areas for particular MPSP types associated with disease occurrence.

Executive summary

Theileriosis is caused by a protozoan that infects red blood cells of cattle, and can result in clinical signs of lethargy, fever, anaemia, jaundice, abortions and mortalities in naive cattle. Many outbreaks of clinical disease have occurred in NSW since 2007, and several have been reported in Victoria in 2011.This project sought to determine the distribution of theilerial types in eastern Australian cattle herds, by survey testing of 516 cattle in 50 herds (NSW 20; Qld 20; south eastern Victoria 10 herds). In the survey testing, PCR analyses of blood samples identified cattle that tested positive on a screening assay for theileriosis, and then confirmed which of the three major types (Ikeda, Chitose, Buffeli) were present in the positive samples. The Buffeli type is not considered to cause disease and has probably been in Australia for centuries, whereas recent outbreaks of theileriosis have been associated with Ikeda type organisms, often with concurrent Chitose infection. In all survey samples, which were derived from herds considered typical of their district and in regions where clinical disease due to theileriosis was infrequent or absent, Chitose was the most prevalent type detected in all three states. However, Ikeda was more limited in distribution. It appeared in three of five regions of Queensland (North, South and South East), and only one region of NSW (North Coast), and was detected in only one animal in Victoria.

In the second part of the study, the significance of the various theilerial types was also examined by testing cattle within seven NSW herds with a history of clinical theileriosis and introductions of cattle. In these herds, Ikeda type was detected in all and Chitose in six. Pale and jaundiced mucosal surfaces were associated with clinically affected groups of cattle, and herds showing numbers of cattle with ≥1% theilerias in their red blood cells on smear examination were associated with high prevalence of Ikeda type, with or without Chitose type. However, for the detection of theilerial infection, PCR testing of EDTA blood was more sensitive than traditional smear examinations, which can be subjective, particularly when infection rates are low.

Infection with Ikeda and Chitose organisms was detected in calves as young as 1-2 weeks, and rapidly increased in prevalence within one month, with Ikeda prevalence increasing at the fastest rate. Direct contact with weaners introduced from coastal areas appeared a risk factor, with high prevalence rates detected in a single time point bleed in purchased adults on one property. Spread within herds appeared variable, and depended on the sampling time point. Cattle without contact with affected mobs may show no evidence of infection, while in others, the lack of direct contact with affected cattle had not prevented infection with the Ikeda type. Within groups showing infection, PCR testing showed repeatedly similar results at multiple samplings at one month intervals, so frequent sampling may be unrewarding once high prevalence is established.

Theilerial infections are long-term and difficult to detect in normal cattle by traditional tests. Smear examinations were found likely to fail to detect infection on repeat sampling as the level of parasites in blood declines. In clinically normal cattle in affected herds with Ikeda type infection, over half of the smear negative animals can still be detected as being infected with the Ikeda type on PCR testing, while 90% of smear positive cases are expected to give PCR positive results for Ikeda type. This shows there is a large reservoir of infected but clinically normal animals in affected herds.

The cattle industry benefits from this work in several ways. Firstly, application of new diagnostic tests was shown to be robust across very large numbers of samples, more sensitive than existing methods and clearly able to detect the major theilerial types. This represents the first structured

survey of cattle herds in the key states where traditional vectors of theilerial strains are known to occur (and where the avirulent Buffeli type organisms may be present). The results indicate that introductions to cattle herds from areas where possible virulent strains of type Ikeda occur can be focussed on one particular region in NSW, and possibly other coastal regions not included in this survey, but involves at least multiple regions in Queensland. In addition it is clear that a very high proportion of young calves and adult cows in an affected herd, or in mobs of cattle that have been in contact with affected or high risk cattle, can be infected with Ikeda and Chitose type organisms, often but not always concurrently. The progress of infection can be rapid, despite the normal clinical appearance of the stock.

Livestock owners and veterinarians can benefit from this knowledge by a better understanding of the epidemiological aspects from a regional perspective and from the herd (infected or high risk) perspective. The work also assists Australian diagnostic laboratories which can provide better tests for producers in defining infection status of different groups of cattle of variable risk status. These assays are important in evaluating animal status when assessing alternate tests, such as serological assays, and the impact of treatment and preventative strategies such as therapeutic drugs or vaccines designed to reduce or eliminate infection.

In addition, the numerous blood samples and DNA extracts characterised in this work will provide a resource of future materials to investigate possible genetic differences between strains of theileria that are currently categorised into the same type by PCR. These samples will also be valuable in improving diagnostic tests in the future, and developing more cost-effective diagnostic assays that can be used to rapidly assess and even quantify the level of infection.

Contents

1 Background

1.1 Benign theileriosis

The term 'benign theileriosis' is used to define infections with certain blood-borne protozoans that are members of a group of organisms sometimes referred to as the *Theileria sergenti/buffeli/orientalis* group. The term 'benign' distinguishes these piroplasms from those responsible for diseases involving lymphocyte transformation and high fatality rates resulting from infections by highly virulent pathogens such as *Theileria parva* and *Theileria annulata.* These two organisms are exotic to Australia, and occur in cattle in areas of east, central and south Africa as East Coast Fever (*T. parva*) or as Tropical Theileriosis in tropical regions of north Africa, southern Europe, China and southern Asia (*T. annulata*), and which are genetically distinct from the benign theilerias (Sarataphan et al., 1999).

Members of the benign theileria group are known to be transmitted by ticks, and have a complex life cycle which involves a stage within red blood cells of cattle where they are referred to as piroplasms. Signs of disease associated with these organisms include lethargy, weakness, fever (>40^{o}C), anaemia, jaundice, mortality and abortions/stillbirths. Known vectors overseas include the bush tick (*Haemaphysalis longicornis*). This is also considered an introduced vector of theilerias in Australia (Callow, 1984), although disputed in favour of *Haemaphysalis humerosa,* a native tick affecting marsupials, by some workers (Stewart et al., 1987). Another likely vector in Australia is *Haemaphysalis bancrofti*, a native tick found on wallabies (Callow, 1984).

The names of the organisms associated with benign theileriosis originated as '*Theileria sergenti'* in Japan (Kawazu et al., 1992; Kubota et al., 1996; Kubota et al., 1995) and this terminology was used to describe isolates in Russia and Korea (Chae et al., 1999; Jeong et al., 2005), but similar organisms, referred to as '*Theileria orientalis'*, were identified in Asia, Europe, the USA and New Zealand. Later workers from Japan also referred to their isolates as '*T. orientalis'* (Ota et al., 2009), probably because the species name '*sergenti*' was deemed appropriate only for sheep-borne parasites (Uilenberg, 2011). In addition, an Australian isolate from Warwick, Qld was given the name '*Theileria buffeli'*, and this organism appeared distinct from those termed '*T. sergenti'* and '*T. orientalis'*, at least in its apparent inability to cause disease. Organisms referred to as *T. buffeli* are widespread, and occur in Africa, Europe, Asia and America. Studies based on DNA sequencing indicated that these organisms were closely related and were not distinct species (Jeong et al., 2010). Together, these organisms are sometimes referred to as the *T. sergenti/buffeli/orientalis* group (Aktas et al., 2006; Fuujisaki, 1992; Jeong et al., 2005; Kakuda et al., 1998; Kim et al., 1998). Based on studies of genes encoding the major piroplasm surface protein (MPSP), three major MPSP types were recognised by specific DNA sequences near the 5' end of the gene that could be targeted by PCR primers. These types were based on reference strains of the organisms and referred to as the Ikeda, Chitose and Buffeli MPSP types. Recently, additional MPSP types have been identified, such as the Thai type (Thailand)(Sarataphan et al., 2003) and Abashiri type (Japan)(Kim et al., 2004).

Recent sequencing studies of the MPSP gene, mainly with overseas isolates, have been applied to classify members of the *T. orientalis/buffeli* group into at least 8 genetic types (or genotypes)(Jeong et al., 2010), with the term *T. sergenti* now considered redundant (Kamau et al., 2011c). In this system, type 1 (Chitose), type 2 (Ikeda) and type 3 (Buffeli) clusters are recognised, with the remaining types 4-8 yet to receive a taxonomic name (Kamau et al., 2011c;

Ota et al., 2009). Further differentiation of strains into genetically-related clusters or types is also possible based on sequencing of other DNA elements such as ribosomal RNA small subunit (SSU) (Kim et al., 2004) or internal transcribed spacer (ITS) genes (Kamau et al., 2011c), and the p23 gene (Ota et al., 2009).

1.2 Occurrence overseas and in Australia before 2007

Benign bovine theileriosis is known to affect cattle in the USA, Europe, Japan, Korea, Africa, China and parts of south east Asia, including Thailand and Indonesia (Aktas et al., 2006; Kakuda et al., 1998). In Australia prior to 2007, organisms of the Buffeli type were known to be present but cause little disease (Callow, 1984). These parasites are suggested to have been introduced with European settlement and adapted to native ticks such as *H. bancrofti* and *H. humerosa* (Kawazu et al., 1992). It is thought that in "enzootic areas" relevant to Buffeli strains, cattle are exposed in early life to organisms of this type, and such exposure may induce immunity. As a result, despite parasitaemias up to 10%, Buffeli type organisms are thought to decline in the blood over several weeks, with blood smears suggesting cattle may have lifelong infections (Callow, 1984).

1.3 Initial NSW outbreaks

During 2007 and 2008, private and district veterinarians were increasingly called to cattle presented with a variety of clinical signs with anaemia as the major clinical finding. In many instances, disease was seen in animals introduced, often in late pregnancy, to coastal properties though disease has also been seen in homebred calves on the coast and all ages on the tablelands. Submissions to veterinary laboratories showed regenerative anaemia and on the basis of blood smear examination diagnosed theileriosis (Izzo et al., 2010); NSW DPI Laboratory records (unpublished). While *Theileria* piroplasms have been seen regularly in blood smears from cattle in NSW, until 2007-8 it had been assumed that the piroplasms were from *Theileria buffeli,* considered a protozoon of low pathogenicity that is spread by *Haemaphysalis* spp ticks (Callow, 1984; Stewart et al., 1987). However it has been suspected that organisms carrying the Major Piroplasm Surface Protein (MPSP) of Chitose type were present in Australia in the 1990's as cattle exported from Australia that developed theileriosis within two weeks of arriving in Japan tested positive (Kubota et al., 1996). When DNA extracts from some of the initial NSW cases were tested in Japan, these showed that organisms with Buffeli, Chitose and Ikeda MPSPs were present (Kamau et al., 2011b). The Ikeda MPSP, often found in combination with other MPSPs such as Chitose, has been associated with significant disease in Japan (Ko et al., 2008; Kubota et al., 1996; Ota et al., 2009; Yokoyama et al., 2011; Zakimi et al., 2006). One recent report has also described similar cases of theileriosis, associated with '*T. orientalis* group organisms' occurring in naïve cattle introduced into a herd in New Zealand (McFadden et al., 2011).

1.4 Impact of disease in Australia

Theileriosis is significant to the cattle industry because disease with associated financial losses is occurring throughout coastal and tableland areas of NSW and recently south east Queensland. Given infection is transmissible, bovine theileriosis has the potential to affect the ability of farmers to trade both domestically and internationally. It is apparent that there are knowledge gaps surrounding theileriosis. In broad terms, we need to understand:

(a) Why the apparent increase in disease has occurred in NSW. Possibilities include (i) a sudden increase in the prevalence of an Ikeda ± Chitose MPSP that was introduced over 100 years ago but has come to prominence with changes to cattle management and/or tick prevalence particularly in coastal areas; (ii) a recent introduction of the Ikeda±Chitose MPSP. At present *Theileria* infection remains an incidental finding throughout most of Queensland and the far north coast of NSW.

(b) Level of significance of theileriosis for the cattle industry. From clinical cases, we know it is widely distributed but its future economic cost to industry cannot be accurately predicted.

(c) How infection is spread. The bush tick, *Haemaphysalis longicornis,* is common on the coast but is rarely seen on tableland cattle. It is responsible for spreading the Ikeda and Chitose strain in Japan. Some Australian workers have been able to demonstrate transmission (Riek, 1982) while others concluded it did not transmit *Theileria* (Stewart et al., 1987).

(d) How effective management or treatment strategies can be provided.

2 Project objectives

2.1 Distribution study

To perform MPSP PCR on 500 samples from cattle in Queensland, the far north coast of NSW, south west NSW and Victoria (contrasting districts where bovine theileriosis is considered endemic, with those where it has not been diagnosed or diagnosed infrequently) and compare to cattle where disease is commonly occurring.

2.2 Significance study

To monitor cattle on at least 6 properties where theileriosis has been diagnosed in an attempt to understand the interplay between parasitaemia, MPSPs and disease.

3 Methodology

3.1 Distribution study

3.1.1 Samples

EDTA blood samples from 516 cattle in 50 Eastern Australian cattle herds were tested in the distribution study, and were derived from 20 herds in various regions of Queensland, 20 herds in various regions of New South Wales and 10 herds in the East Gippsland and Wellington districts of Victoria (Table 1). The samplings were chosen as a random selection and considered representative of normal cattle in these districts by the District Veterinarians/Veterinary Officers from those districts. In addition, the regions sampled were specifically chosen because the prevalence of clinical disease was considered low or not reported. For this reason, certain regions within NSW (e.g. Mid-Coast and Cumberland) were excluded. Information on animal types and ages in the sampled herds for most NSW and all Victorian herds was available and is described in Table 2. Limited information was available from Queensland herds.

3.1.2 Clinical pathology

Where fresh samples were obtained, packed cell volume (PCV) and blood smear examinations were undertaken. Smears were stained by modified Giemsa (Diff-Quik) stain and results were recorded according to red blood cell morphology and staining. Erythrocyte abnormalities such as anisocytosis (variable size), poikilocytosis (variable shape), polychromasia (variable staining), Howell-Jolly bodies and nucleated reds were recorded. The presence of intraerythrocytic theilerial forms and the proportion of red cells showing theilerial-like organisms in smears were also recorded.

3.1.3 PCR assays

Blood samples were tested in conventional PCR assays for the detection of the major piroplasm surface protein (MPSP) relevant to the p32, Ikeda, Chitose and Buffeli MPSP genes, using primers and PCR conditions as described by Zakimi et al (Zakimi et al., 2006), with the following modifications:

1. DNA extracts were produced using a commercial kit (DNEasy, Qiagen) according to the manufacturer's instructions for blood samples
2. To overcome possible inhibition to the PCR reaction from blood components, DNA extracts were tested neat and at a 1:10 dilution in sterile MilliQ water
3. All PCR reactions were undertaken using 50µL reaction volumes and using 2µL of DNA template (for neat and 1:10 diluted extract)
4. All PCR reagents used were from a single commercial source (Roche)
5. Two positive PCR control extracts, one negative MilliQ water control and one extraction process control were tested with each test PCR run
6. PCR testing was performed on Corbett Research 960 thermocyclers (models 960C, 960G and FTS 960)
7. Amplified product was run in 1% agarose gel (Agarose low EEO, AppliChem GmbH, Darmstadt, Germany) in 0.5X Tris-buffered EDTA (Amresco TBE buffer 10X, Amresco, Solon, Ohio, USA) after electrophoresis in 120 mL gels loaded with 15 µL of PCR product for samples and 3 µL molecular weight standards (100 bp DNA ladder, MBI Fermentas) with electrophoresis at 94V for 1.5 h
8. Amplified product was visualised and recorded using a BioRad Gel Doc 2000 system.

The cocktail mixture was as described in Table 3. Valid tests had controls showing a positive gel band at the location as described in Table 4. PCR results were recorded for each test according to Table 5.

In the distribution study, the p32 assay was used as a screening assay in most herds. Samples that tested negative in the p32 PCR at both the neat and 1:10 dilutions of DNA extract were generally not further tested and interpreted as test negative for theileriosis. However, in two NSW herds tested toward the end of the study (N16-17, Table 10), samples that tested negative in the p32 PCR were additionally tested in the MPSP type specific assays.

Table 1. Source of samples for Theileria distribution study

Qld herds	District - Shire or Centre	Location	No. samples = 222
Q1	South – Dalby	Wandoan	10
Q2	North – Townsville	Oonoonba	10
Q3	South East - Scenic Rim	Mt Walker	11
Q4	South – Balonne	St George	16
Q5	West – Murweh	Morven	10
Q6	South – Toowoomba	Warwick	12
Q7	South East - Moreton Bay	Dayboro	8
Q8	South East - South Burnett	Murgon	14
Q9	Central – Mackay	Koumala	10
Q10	Central – Mackay	Crediton	10
Q11	North – Tablelands	Mareeba	10
Q12	North – Tablelands	Mareeba	10
Q13	South East – Gatton	Gatton	10
Q14	South East – Gatton	Gatton	10
Q15	South East - Gold Coast	Numinbah Valley	12
Q16	South – Dalby	Moonk	10
Q17	South – Dalby	Wuranga	10
Q18	South East - South Burnett	Murgon	10
Q19	South East – Gympie	Gympie	20
Q20	South East - Fraser Coast	Maryborough	9
NSW herds	**District**	**Location**	**No. samples = 194**
N1	North West - Warialda/Moree	Wallangra	10
N2	Hume – Gundagai	Willowglen	10
N3	South East – Cooma	Lake George	10
N4	Lachlan – Forbes	Forbes	10
N5	North West - Warialda/Moree	Bingara	12
N6	Central West – Dubbo	Dubbo	16
N7	North West - Warialda/Moree	Warialda	10
N8	Tablelands – Goulburn	Currawang	10
N9	Hume – Lavington	Boongarra	10
N10	North Coast – Grafton	Lawrence	10
N11	North Coast – Casino	Sawpit Creek	5
N12	North Coast – Lismore	Chillingham	5
N13	North Coast- Casino	Dobies Bight	11
N14	South East – Braidwood	Craigie	10
N15	South East – Cooma	Dalgety	4
N16	Hume - Wagga Wagga	Mangoplah	10
N17	Hume - Wagga Wagga	Wagga Wagga	11
N18	Riverina – Deniliquin	Finley	10
N19	Riverina – Hay	Wangara	10
N20	Riverina – Hay	Wanda Park	10
Victorian herds	**District**	**Location**	**No. samples = 100**
V1	East Gippsland	Bruthen	10
V2	East Gippsland	Bengworden	10
V3	East Gippsland	Orbost	10
V4	East Gippsland	Clifton Creek	10
V5	East Gippsland	Marlo	10
V6	East Gippsland	Jarrahmond	10
V7	East Gippsland	Cabbage Tree Creek	10
V8	East Gippsland	Cann River	10
V9	Wellington	Welshpool	10
V10	Wellington	Hunterston	10

Addendum to Table 1.
Location of herds in Queensland (A), New South Wales (B) and Victoria (C) for distribution study.
Queensland DPI regions and NSW LHPA districts are shown on their respective maps. In Victoria, the two western herds are in the Wellington district and the remainder are in the East Gippsland district.

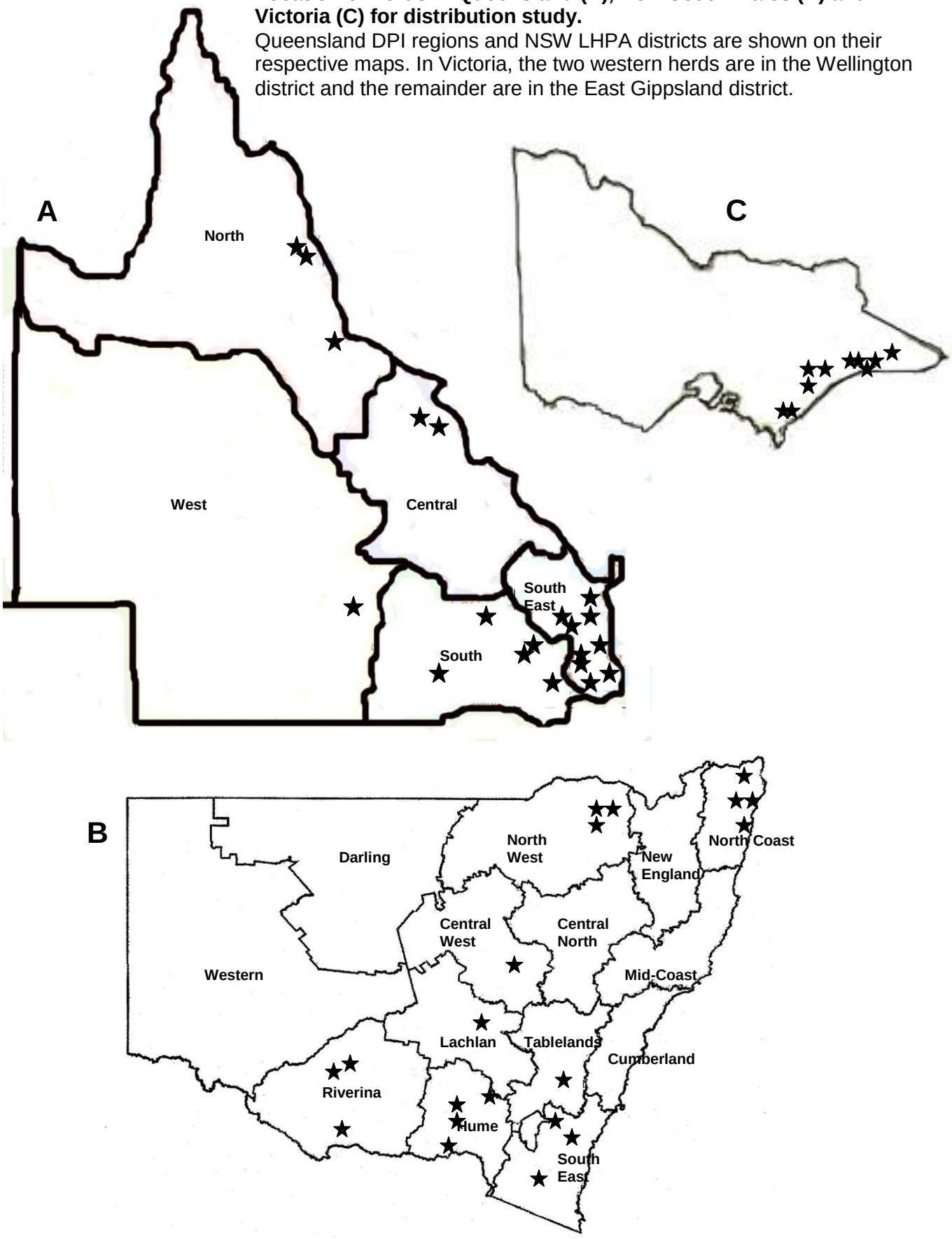


Table 2. Herd information for herds tested in distribution study

Herd	Type	Breed	Age	Sex
Q1	Beef	Crossbred	6-10 mo	Not specified
Q2	Beef	Droughtmaster	Not specified	Not specified
Q3	Dairy	Illawarra	Not specified	Not specified
Q4	Beef	Charolais	Not specified	Not specified
Q5	Beef	Brangus	6-10 mo	Not specified
Q7	Dairy	Friesian	Not specified	Female
Q16	Beef	Angus	2-3 yr	Female
Q17	Beef	Droughtmaster	18 mo	Male
Q18	Beef	Not specified	Not specified	Not specified
Q20	Beef	Brahman	1-2 yr	Female
Q6,Q8-15, Q19	Not specified	Not specified	Not specified	Not specified
N1	Beef	Angus	2 yr	Female
N2	Beef	Angus/Murray Grey	2-6 yr	Female
N3	Beef	Angus	2 yr	Female
N4	Dairy	Friesian	Various	Female
N5	Beef	Shorthorn	2 yr	Female
N6	Beef	Angus	10-11 mo	Mixed
N7	Beef	Hereford	2 yr	Female
N8	Beef	Hereford	2 yr	Female
N9	Beef	Hereford	3 yr	Female
N10	Mixed	Mixed	Adult	Female
N11	Beef	Braford	18 mo	Steers
N12	Beef	Santa Gertrudis	Various	Mixed
N13	Beef	Braford	2-6 mo	Mixed
N14	Beef	Hereford	14 mo	Steers
N15	Beef	Hereford	Mixed	Female
N16	Beef	Droughtmaster	Adult	Female
N17	Beef	Angus	10 mo	Female
N18	Dairy	Mixed	Adult	Female
N19	Beef	Angus	11 mo	Female
N20	Beef	Angus	5-6 yr	Female
V1, V4-V8	Dairy	Friesian and Jersey	2 yr and over	Female
V2-V3, V9-V10	Dairy	Friesian	2 yr and over	Female

Table 3. Volume of reagents in PCR mixture

PCR mix	Volume per sample (µL)
Primer F	1.25
Primer R	1.25
dNTP (Roche)	5
PCR buffer [Roche with	5
$MgCl_2$ (Roche)	0.5
1U Taq (Roche)	0.25
H_2O	34.75
Vol. PCR mix per tube	48
Template	2

Table 4. Size of expected PCR products for various MPSP PCR assays

PCR assay	Product size
p32	875 bp
Ikeda	826 bp
Chitose	831 bp
Buffeli	826 bp

Table 5. Interpretation of PCR assay results

Result	Abbreviation	appearance
positive	pos	strong, definite band matching the control in size
weak positive	wkpos	correct size but much weaker than control, definite line
very weak positive	vwkpos	correct size but only just visible
negative	neg	no bands evident corresponding to control

3.2 Significance study

3.2.1 Samples

485 EDTA blood samples from seven NSW beef herds in the Mid-Coast (two herds), Tablelands (one herd), Hume (three herds) and Lachlan (one herd) Livestock Health and Pest Authority (LHPA) districts (Tables 6, 7) were examined by PCR and for evidence of abnormalities in clinical pathological parameters. Sampling was undertaken on one occasion in two of the herds (herds W, B), on two occasions in 3 herds (herds R, Ge, Ga) and on up to 4 occasions in the remaining two herds (herds Pa, Pe) at intervals of approximately 1 month (Table 7). In some herds multiple mobs of cattle were examined on single or multiple occasions. In herds with multiple bleeds, selected animals were bled on consecutive occasions.

The ages of the sampled animals varied from 1 month old calves to adults (Table 6) and the number sampled from separate groups on each farm generally numbered between 10 and 20 animals.

The herds were selected with the assistance of District Veterinarians who also undertook the sampling, and were based on criteria outlined in Table 8, such as:

i. prior herd history of clinical theileriosis in last 1-8 months
ii. variable history of introductions, and sampling of recent introductions if possible
iii. differentiation of subgroups that were in contact with clinically affected stock, were cohorts of affected stock or had no contact with affected stock

Table 6. Source of samples for significance study

Herd	Location	District	Type	Breed	Sex
Pa	Huntington	Mid Coast - Kempsey	Beef	Angus	Mixed
W	Byng	Tablelands	Beef	Angus and Angus cross	Female
Pe	Pee Dee	Mid Coast - Kempsey	Beef	Angus	Female
R	Ournie	Hume - Lavington	Beef	Angus	Female
Ge	Monteagle	Lachlan	Beef	Angus and Murray Grey	Female
B	Adelong	Hume - Wagga Wagga	Beef	Mixed	Mixed
Ga	Holbrook	Hume - Wagga Wagga	Beef	Angus and Hereford	Female

Table 7. Sampling dates and details for significance study

Herd	Sample dates (mo/yr)	Sampling	Age	No. sampled
Pa	11/2010	Bleed 1 calves	1-2 weeks	10
	12/2010	Bleed 2 calves	1-2 months	10
	2/2011	Bleed 3 calves; Bleed 1 adults	2-3 months, 2 years	10,18
	4/2011	Bleed 4 calves; Bleed 1 calves of heifers bled 2/2011	3.5-4.5 months, < 1 month	10,17
W	12/2010	Bleed 1	Adult	27
Pe	2/2011	Bleed 1	2-3 yr	38
	3/2011	Bleed 2	2-3 yr	38
	4/2011	Bleed 3	2-3 yr	38
	4/2011	Bleed 4	2-3 yr	38
R	5/2011	Bleed 1	2 yr	50
	6/2011	Bleed 2	2 yr	30
Ge	4/2011	Bleed 1	Adult	17
	5/2011	Bleed 2	Adult	15
B	5/2011	Bleed 1	Mixed	50
Ga	6/2011	Bleed 1	Mixed	34
	7/2011	Bleed 2	Mixed	34
			TOTAL	485

Table 8. Herd histories for significance study

Herd	Prior Theilerial infection status (mo/year)	References	Cattle movements
Pa	Theileria mortality 6/2010 in bull purchased 6 weeks earlier. Then mortality with regenerative anemia in 3 month old calf 10/2010 with tick burden and 5% erythrocytes with theilerias. Ikeda and Chitose types confirmed in calf, and Ikeda type in a new bull 10/2010. Bull smear positive 12/2010 with 13% erythrocytes containing theilerias.	M10-13607	Initial bull ex Coonamble 4/2010. New bull ex Rylstone 2 weeks prior to 10/2010.
W	Four clinical cases in cows with regenerative anaemia and theilerias on smears 11/2010. Ikeda, Chitose and Buffeli types detected. All clinicals originated from inland properties, and were likely to be incubating disease on arrival.	M10-14951	Introduction of 17 cows with calves 10/2010. 14 were inland cattle and 3 Gloucester cattle, all initially assembled at Young 9/2010, went to Gloucester 10/2010, thence to saleyards and direct to herd W 10/2010. The 14 inland cattle had various movements in 2006-2010 from or via Moree, Wanaaring, Qld, Milparinka, Cooma then through Gundagai, Wagga or direct to Young.
Pe	Animals in coastal herd; possible risk since movements between coast and Gunnedah and exposure of non-coast-born cattle.		Introduced 38 cows ex Gunnedah 2/2011, of which 13/38 born on this coastal property.
R	Two clinical 2.5 yo cows with regenerative anaemia and 3-5% theileria 4/2011 confirmed Ikeda positive. Three suspect cases with marginal regenerative anaemia 5/2011 confirmed Ikeda positive.	M11-06316 M11-06671	Heifers introduced 9/2010 after agistment at second property on river flats. Neighbour to that property had introductions. No other introductions except bulls ex WA.
Ge	Mortality and two clinical cases with pale mucosae and lethargy in introduced cows (1-8 weeks in herd) 4/2011. Confirmed case with anaemia and moderate theilerias on smears, no PCR performed.	M11-05114	Introduced 10 head 3/2011 and 10 head 2/2011. Introductions ex Young, Wagga, Gundagai, Narrandera and Cooma, but included 3 cattle originally ex Qld then in Gundagai for 1-3 years. Two cattle came via Brewarrina to Gundagai, including one of the ex Qld cows.
B	Clinically affected cattle showing jaundice in one mob 5/2011. Confirmed with theileria on smears and Ikeda ± Chitose positive.	M11-07733	Cattle in three mobs on 300 acres. One affected, one purchased cattle mixed with homebred weaners derived from affected mob, one no direct contact with other mobs. Weaners had returned from coastal agistment at Taree.
Ga	Six mortalities in mob of 30 resident adult cattle by 12/2010. Suspect clinical case with regenerative anaemia and low numbers of theilerias on smear 11/2010. Ikeda detected. Additional deaths in resident cattle attributed to theileriosis between 11/2010 and 5/2011.	M10-14748	100 weaners introduced ex Armidale 5/2010 and removed 6/2010. 129 weaners introduced ex Taree 7/2010 and removed 8/2010. No clinical disease in either group of weaners.

3.2.2 Clinical examinations and clinical pathology

Cattle were examined clinically by field veterinarians collecting the blood samples, who recorded rectal temperatures and the appearance of the mucous membranes. The latter were compared among herds on the following scale: pink (normal), pale pink, pale, or jaundiced. EDTA blood samples were tested for packed cell volume (PCV) and thin blood smears were examined for erythrocyte morphology and presence of theilerial forms. The latter were quantified as the percentage of erythrocytes that contained theilerial forms. Results for body temperature were compared among herds by determining the proportion of animals with temperatures below 40^{o}C and the proportion with temperatures of 40^{o}C or higher, since the upper normal range for cattle over 12 months is 39.2^{o}C and under 12 months is 39.8^{o}C (Kelly, 1974).

3.2.3 PCR assays

All bloods were subjected to PCR assays for the p32, Ikeda, Chitose and Buffeli MPSP genes, and DNA extracts were tested separately at neat and a 1:10 dilution in sterile water, as described for the distribution study. Samples were tested in the type specific assays regardless of their p32 PCR result. To compare the strength of PCR reactivity at different bleeds, the strength of specific gel bands as described in Table 5 were scored at each dilution tested, on a scale from 0 to 3 (0 = negative; 1 = very weak positive, 2 = weak positive; 3 = positive). For each sample, the highest score at either dilution of DNA extract was taken as the final result, and the mean PCR score (± sem) per group sampled from each herd at each bleed was determined. The proportion of PCR reactors from these same groups was also determined, where a reactor on any given test was interpreted as an animal with a score of 1 or more in testing of either DNA extract dilution.

4 Results

4.1 Distribution study PCR results

4.1.1 Queensland herds

Among the 20 Queensland herds, PCR testing detected 17 herds (85%) with theilerial infection and almost 57% of samples tested were positive in the p32 PCR (Table 9). Specific MPSP types Ikeda, Chitose and Buffeli were found in these samplings, with Chitose (44%) being the most prevalent followed by Buffeli (25%) and Ikeda (11%) (Table 9). MPSP type Chitose was found in 16 /20 Queensland herds (80%), Buffeli in 14/20 (70%) and Ikeda in 7/20 (35%) (Table 9).

Across the various regions in Queensland, Ikeda type was only detected in 3/5 districts, although sampling in the remaining two districts was limited to 1-2 herds (Table 10). A higher prevalence of Ikeda infection was found in the South-East and North regions (15-20% in each) while the South region showed a lower prevalence (3%) in sampled herds. While approximately half the herds in the South-East (4/9) and North regions (2/3) showed evidence of Ikeda infection, the within-herd prevalence was very variable. Of the 16 confirmed Ikeda samples in the South-East, 12 were associated with a single herd (Q15), while the three other Ikeda-infected herds had only 1-2 positive blood samples detected from 8-14 animals sampled per herd. Likewise, of the 6 confirmed Ikeda samples from North region herds, 5 of these were derived from a single herd (Q11) while the second Ikeda-positive herd (Q12) showed only 1/10 samples as positive for this MPSP type (Table 10).

In Queensland, MPSP type Chitose was widespread with 4/5 regions showing PCR positive results for this type, and regional prevalences for sampled animals between 22-70% (Table 10). The Buffeli MPSP type showed a lower and more uniform prevalence (17-40%) among these four districts.

Table 9. PCR reactivity among 20 Qld herds using a screening PCR (p32), and three type-specific PCRs for MPSP types Ikeda (I), Chitose (C) and Buffeli (B)

Herd	Samples	P32 pos[1]	I pos	C pos	B pos
Q1	10	10	0	9	5
Q2	10	10	0	10	3
Q3	11	7	0	4	5
Q4	16	0	NT[2]	NT	NT
Q5	10	0	NT	NT	NT
Q6	12	8	2	3	5
Q7	8	6	1	7	4
Q8	14	14	2	12	3
Q9	10	7	0	9	2
Q10	10	6	0	5	3
Q11	10	8	5	2	0
Q12	10	10	1	9	9
Q13	10	7	0	2	0
Q14	10	1	0	0	1
Q15	12	11	12	5	2
Q16	10	0	NT	NT	NT
Q17	10	1	0	1	0
Q18	10	8	1	7	3
Q19	20	6	0	6	4
Q20	9	6	0	6	6
Total	**222**	**126**	**24**	**97**	**55**
	%	**56.8%**	**10.8%**	**43.7%**	**24.8%**

[1] pos: positive result in PCR assay

[2] NT: not tested when samples negative on p32 screening assay

Table 10. PCR reactivity among Qld districts showing relative proportions of samples per district that tested PCR positive using a screening PCR (p32), and three type-specific PCRs for MPSP types Ikeda (I), Chitose (C) and Buffeli (B). Percentage figures indicate district prevalence.

District	Herds	Herd IDs	Samples	P32 pos[1]	I pos	C pos	B pos
North	3	Q2, Q11, Q12	30	28 (93.3%)	6 (20%)	21 (70%)	12 (40%)
Central	2	Q9, Q10	20	13 (65%)	0	14 (70%)	5 (25%)
West	1	Q5	10	0	NT[2]	NT	NT
South	5	Q1, Q4, Q6, Q16-17	58	19 (32.8%)	2 (3.4%)	13 (22.4%)	10 (17.2%)
South East	9	Q3,Q7-8, Q13-15, Q18-20	104	66 (63.5%)	16 (15.4%)	49 (47.1%)	28 (26.9%)
Total	20		**222**	**126**	**24**	**97**	**55**

[1] pos: positive result in PCR assay

[2] NT: not tested when samples negative on p32 screening assay

4.1.2 NSW herds

In NSW, 9/20 herds gave p32 positive results, although an additional herd (herd N16), when further tested using specific MPSP type PCRs, also revealed Chitose type in one animal (Table 11). MPSP type Ikeda was detected in 4/20 herds (20%), Chitose in 8/20 (40%) and Buffeli in 4/20 (20%) (Table 11). On a Livestock Health and Pest Authority (LHPA) district basis, NSW results showed Ikeda was only detected in one district (North Coast) among 8 districts sampled (Table 12). Within the North Coast samplings from four herds, all four showed this MPSP type in the majority of samples from each (9/10, 5/5, 3/5, 6/11) (Table 12).

MPSP types Chitose and Buffeli in NSW herds by region showed a strong predilection to the North Coast and North West districts (Table 12), with an even distribution of both types at approximately 45% prevalence in the North West and a higher Chitose than Buffeli presence on the North Coast (65% vs 19% respectively). Buffeli was not detected in the other regions, while Chitose appeared at low prevalence (2-10%) in three of the other regions (Table 12).

Table 11. PCR reactivity among 20 NSW herds using a screening PCR (p32), and three type-specific PCRs for MPSP types Ikeda (I), Chitose (C) and Buffeli (B)

Herd	Samples	p32 pos[1]	I pos	C pos	B pos
N1	10	6	0	4	6
N2	10	0	NT[2]	NT	NT
N3	10	0	NT	NT	NT
N4	10	4	0	1	0
N5	12	0	NT	NT	NT
N6	16	0	NT	NT	NT
N7	10	9	0	10	9
N8	10	0	NT	NT	NT
N9	10	0	NT	NT	NT
N10	10	9	9	10	0
N11	5	5	5	5	4
N12	5	4	3	5	2
N13	11	6	6	0	0
N14	4	0	NT	NT	NT
N15	10	2	0	0	0
N16	11	0	0	1	0
N17	10	0	0	0	0
N18	10	1	0	1	0
N19	10	0	NT	NT	NT
N20	10	0	NT	NT	NT
Total	**194**	**46**	**23**	**37**	**21**
	%	**23.7%**	**11.9%**	**19.1%**	**10.8%**

[1] pos: positive result in PCR assay [2] NT: not tested when samples negative on p32 screening assay

Table 12. PCR reactivity among NSW districts showing relative proportions of samples per district that tested PCR positive using a screening PCR (p32), and three type-specific PCRs for MPSP types Ikeda (I), Chitose (C) and Buffeli (B). Percentage figures indicate district prevalence.

District	Herds	Herd IDs	Samples	p32 pos[1]	I pos	C pos	B pos
North Coast	4	N10-N13	31	24 (77.4%)	23 (74.2%)	20 (64.5%)	6 (19.4%)
North West	3	N1, N5, N7	32	15 (46.9%)	0	14 (43.8%)	15 (46.9%)
Central West	1	N6	16	0	NT[2]	NT	NT
Lachlan	1	N4	10	4 (40%)	0	1 (10%)	0
Tablelands	1	N8	10	0	NT	NT	NT
South East	3	N3, N14-15	24	2 (8.3%)	0	0	0
Hume	4	N2, N9, N16-17	41	0	0	1 (2.4%)	0
Riverina	3	N18-20	30	1 (3.3%)	0	1 (3.3%)	0
Total	20		**194**	**46**	**23**	**37**	**21**

[1] pos: positive result in PCR assay

[2] NT: not tested when samples negative on p32 screening assay

4.1.3 Victorian herds

In Victorian samplings (Tables 13, 14), 34% of samples and 8/10 herds showed evidence of theileria on p32 assays, but these were confirmed by MPSP type specific PCRs to be predominantly Chitose and Buffeli type infections (Table 9). One Victorian herd (V3) from East Gippsland had evidence of Ikeda MPSP type, in a single sample from that herd (Tables 13, 14).

On a regional basis, theilerial infections were not detected in either of the Wellington herds, but were found in 42% of samples from East Gippsland and in all 8 herds sampled (Tables 13, 14). The Ikeda prevalence was very low in that district (1%), and the prevalence of Chitose and Buffeli types were similar and were found in approximately 30% of samples from the 8 East Gippsland herds.

Table 13. PCR reactivity among 10 Victorian herds using a screening PCR (p32), and three type-specific PCRs for MPSP types Ikeda (I), Chitose (C) and Buffeli (B)

Herd	Samples	p32 pos[1]	I pos	C pos	B pos
V1	10	2	0	0	2
V2	10	2	0	0	2
V3	10	1	1	1	0
V4	10	1	0	1	0
V5	10	7	0	6	4
V6	10	8	0	6	7
V7	10	6	0	4	4
V8	10	7	0	7	3
V9	10	0	NT	NT	NT
V10	10	0	NT	NT	NT
Total	**100**	**34**	**1**	**25**	**22**
	%	**34.0%**	**1.0%**	**25.0%**	**22.0%**

[1] pos: positive result in PCR assay

[2] NT: not tested when samples negative on p32 screening assay

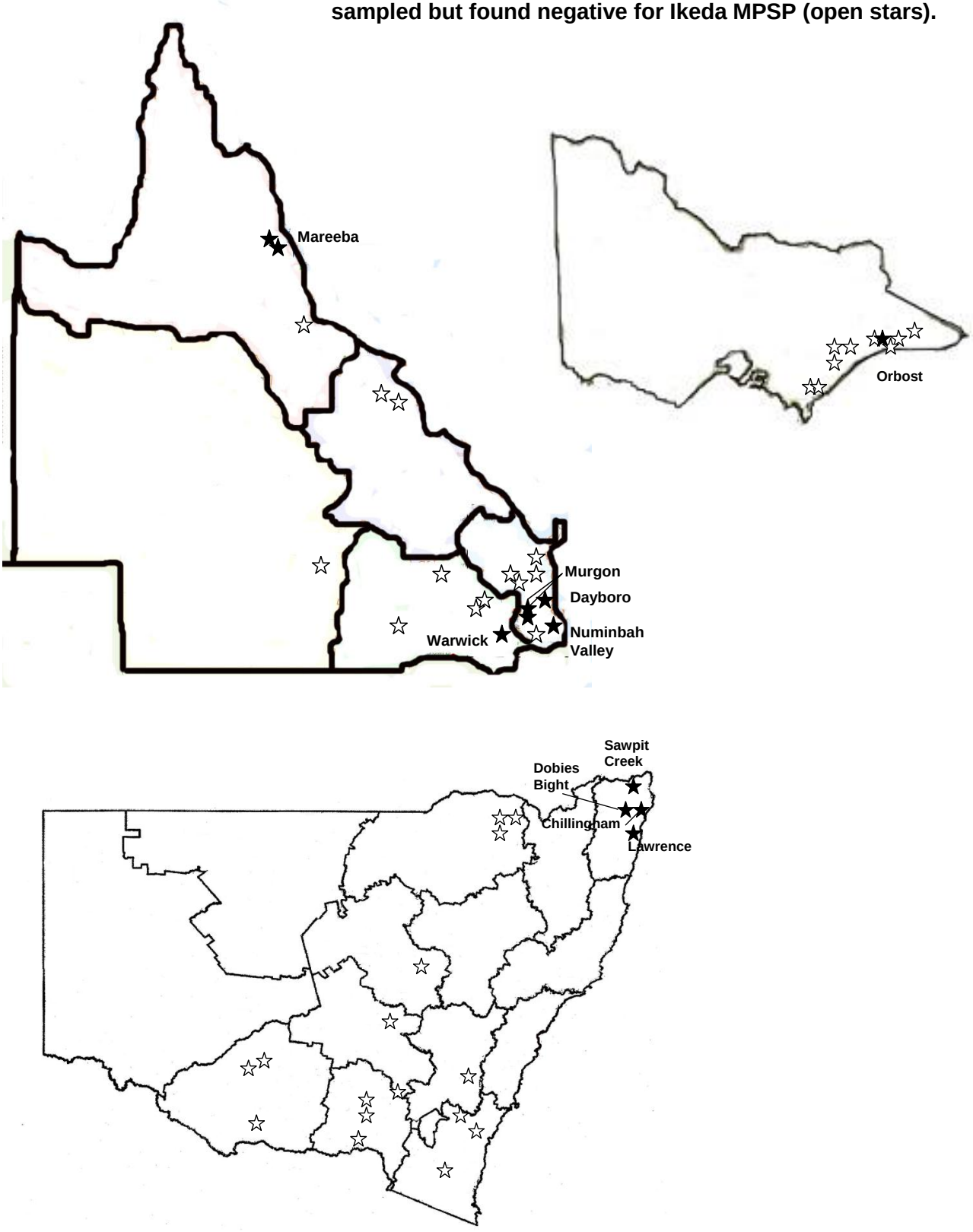
Table 14. PCR reactivity among two Victorian districts showing relative proportions of samples per district that tested PCR positive using a screening PCR (p32), and three type-specific PCRs for MPSP types Ikeda (I), Chitose (C) and Buffeli (B). Percentage figures indicate district prevalence.

District	Herds	Herd IDs	Samples	p32 pos[1]	I pos	C pos	B pos
East Gippsland	8	V1-8	80	34 (42.5%)	1 (1.3%)	25 (31.3%)	22 (27.5%)
Wellington	2	V9-10	20	0	NT	NT	NT
Total			**100**	**34**	**1**	**25**	**22**

[1] pos: positive result in PCR assay

[2] NT: not tested when samples negative on p32 screening assay

State maps showing locations of herds detected as PCR positive for Ikeda MPSP (filled stars), compared with herds sampled but found negative for Ikeda MPSP (open stars).



4.2 Distribution study clinical pathology results

Results of clinical pathology derived from 28 of the 30 herds surveyed in NSW and Victoria showed very few animals sampled were anaemic (PCV < 24). Such animals, when found, were only evident in herds N6 and V7 (Table 15). Among these herds, smear examinations revealed theilerial forms in eryrthocytes in only one herd (N13), where 4/10 animals were positive on smears and also positive by PCR. The four smear positive cattle had an estimated prevalence of theilerial forms in erythrocytes of <1% (3 animals) to 2% (1 animal), and all were p32 PCR positive and three of the four were also positive for the Ikeda MPSP. None of the smear positive cases showed any abnormalities in erythrocyte shape, size or staining, nor inclusion bodies or increased nucleated red cells.

Table 15. Clinical pathology in NSW and Victorian herds, with comparative PCR positive screening assay (p32) results. Herds with shaded cells were PCR positive for Ikeda type.

Herd ID	Samples	PCV range	Smears positive for Theileria	p32 PCR positive
N1	10	33-38	0	6
N2	10	35-40	0	0
N3	10	30-44	0	0
N4	10	27-36	0	4
N5	12	29-36	0	0
N6	16	19-31	0 (12 smears); 4 unsuitable	0
N7	10	33-46	0	9
N8	10	35-41	0	0
N9	10	35-42	0	0
N10	10	27-41	0	9
N11	5	28-35	0	5
N12	5	NT[1]	NT	4
N13	11	33-49	4	6
N14	10	30-44	0	0
N15	4	37-42	0	2
N16	10	35-45	0	0
N17	11	27-37	0	0
N18	10	NT	NT	1
N19	10	38-46	0	0
N20	10	28-46	0	0
V1	10	24-31	0	2
V2	10	28-37	0	2
V3	10	29-33	0	1
V4	10	32-39	0	1
V5	10	27-34	0	7
V6	10	30-35	0	8
V7	10	21-34	0	6
V8	10	34-44	0	7
V9	10	24-36	0	0
V10	10	30-38	0	0

[1] NT: not tested

4.3 Significance study PCR results

The PCR results from the significance study are presented in a series of figures (Figures 1-7) that demonstrate the trends found in each of the seven study herds.

Results in Herd Pa (Mid-Coast LHPA; Figure 1) demonstrated the increase in reactivity among calves in an infected herd as they age from 1-2 weeks to 4.5 months, at which time 100% of calves were infected with almost maximum positive PCR readings for all 3 MPSP types. However the incidence of infection differed between the 3 MPSP types, with MPSP type Buffeli appearing much slower than the others, and type Ikeda the fastest. In this herd, 100% infection by Ikeda type was evident at 1-2 months of age, and this high rate of infection was maintained (90-100% of calves with strong reactivity on PCR) until 4.5 months of age. The adult herd showed almost 90% were PCR positive for benign theileriosis, but Ikeda reactivity in their calves at less than 1 month of age was higher in terms of reactivity strength and percentage of test positive animals. There was a difference in Ikeda prevalence of <70% for cows to almost 90% for their calves. In contrast, the strength and prevalence of the two other MPSP types (Chitose and Buffeli) was lower in the calves compared to their dams.

Herd W (Tablelands) showed a marked difference in prevalence between homebred and introduced stock, with the former being PCR negative and the latter indicating a prevalence of 94% for benign theileriosis, with types Ikeda and Chitose being present with similar prevalence (>80%) and with similar strength of reactivity (Figure 2).

Herd Pe (Mid-Coast) involved consecutive testing of 38 introduced cattle on four occasions, 1 month apart. The prevalence of benign theileriosis on all occasions was ≥ 95%, and reactivity to all MPSP types was present, and each MPSP type remained at a similar prevalence and strength of reactivity on each occasion (Figure 3). Therefore this herd appears to have already stabilised in its infection status within two weeks after introduction to this coastal property from a property in the northern tablelands.

Herd R (Hume) contained animals with a known theileriosis-infected mob of two year old cattle that showed a pure Ikeda infection at a prevalence of 50%. Two year old cohorts in two separate mobs that had not shown clinical signs of infection, similarly revealed evidence only of Ikeda infection but at a lower prevalence of approximately 10% (Figure 4) and similar low mean reactivity for the two groups. Some increase in Ikeda prevalence (to 20%) was found in one cohort group. A separate three year old group without any contact with the two year old infected mob showed no evidence of benign theileriosis, suggesting spread on the property by vectors had not been efficient.

The results in Herd Ge (Lachlan) were similar to findings in adults in herds Pa and Pe, but with lower overall prevalence of types Ikeda and Chitose, and a predominance of type Ikeda at both samplings 1 month apart (approximately 80% infected)(Figure 5). The pattern between bleeds was almost identical, indicating a stable infection status had been reached, and there was a very low prevalence of the Buffeli type (<10%) on both occasions.

Herd B (Hume) was a single bleed comparing infection rates between mobs that had been either clinically affected, or were a group of purchased cattle mixed with introduced coastal weaners, or were a group that had no contact with clinically affected cattle. In contrast with results in herd R,

60% of the non-contact cattle in this herd had evidence of infection with benign theileriosis types, with Ikeda predominant and infecting 50% of the non-contact group (Figure 6). This herd also showed a higher prevalence and mean PCR reactivity score among the mixed group (90% Ikeda infection rate) than the mob currently containing cattle that had shown clinical evidence of benign theileriosis (70% Ikeda infection rate). The mixed group also had a very high prevalence of Chitose type (90%) compared with the clinically affected and non-contact animals (10% each), as well as higher Buffeli infection rates and reactivity than the remaining two groups (Figure 6).

In herd Ga (Hume) where sampling was undertaken several months after evidence of clinical disease associated with Ikeda type infection (Table 7), this remained the predominant MPSP type in the herd at consecutive samplings 1 month apart, and showed a similar strong mean PCR reactivity and moderately high prevalence (approximately 65%) on both occasions (Figure 7). This resembled the infected group sampled on Herd R (Figure 4), but was at higher infection prevalence.

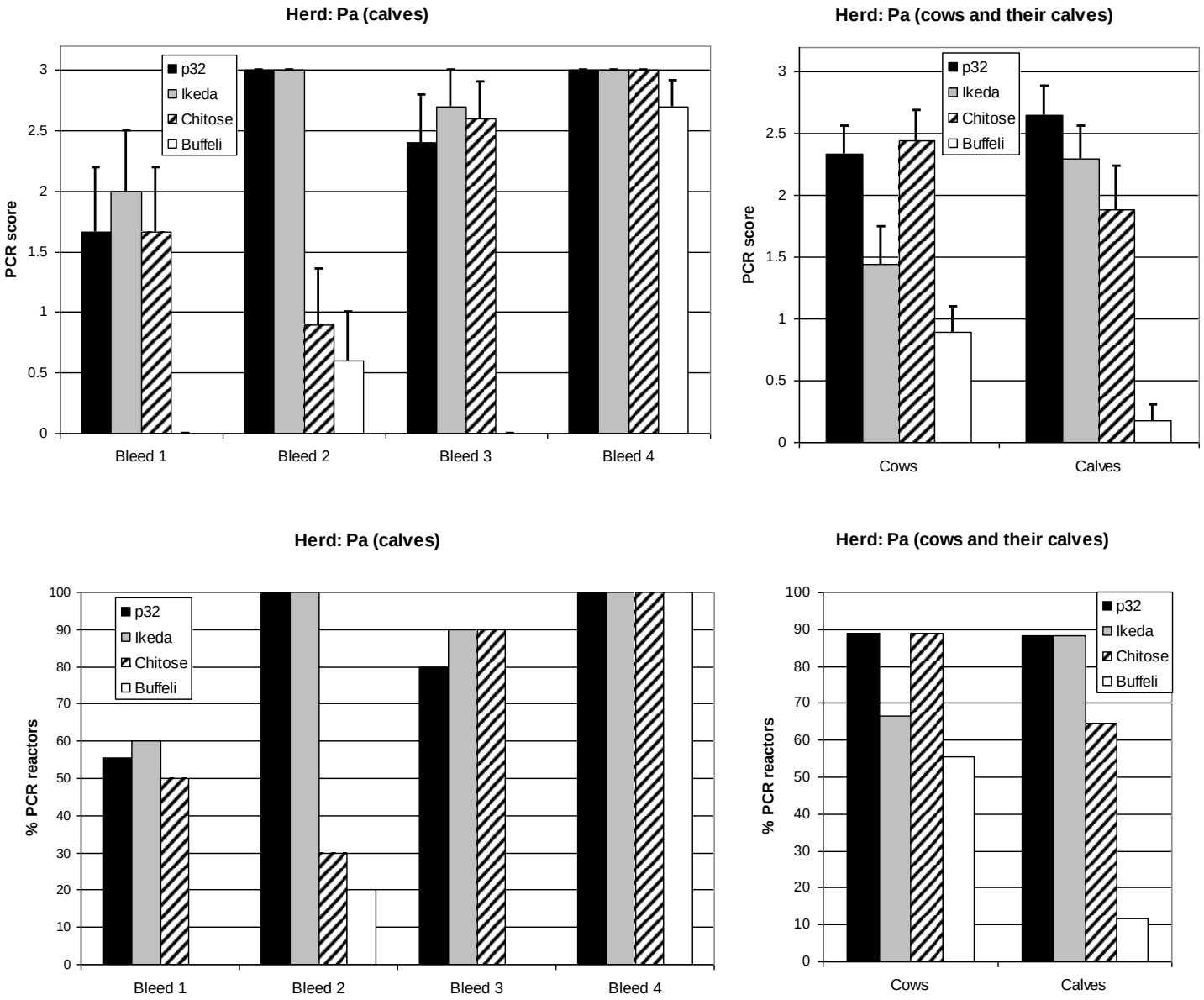


Figure 1. PCR reactivity in Herd Pa (Mid-Coast). In this herd, four consecutive bleeds in 10 calves and a single bleed of 18 adults and 17 of their calves were assessed. PCR reactivity is measured as a percentage of positive reactors among all animals tested, or as the mean PCR score in a range of 0-3 (where 0 = negative; 1 = very weak positive; 2 = weak positive; 3 = positive) ±sem

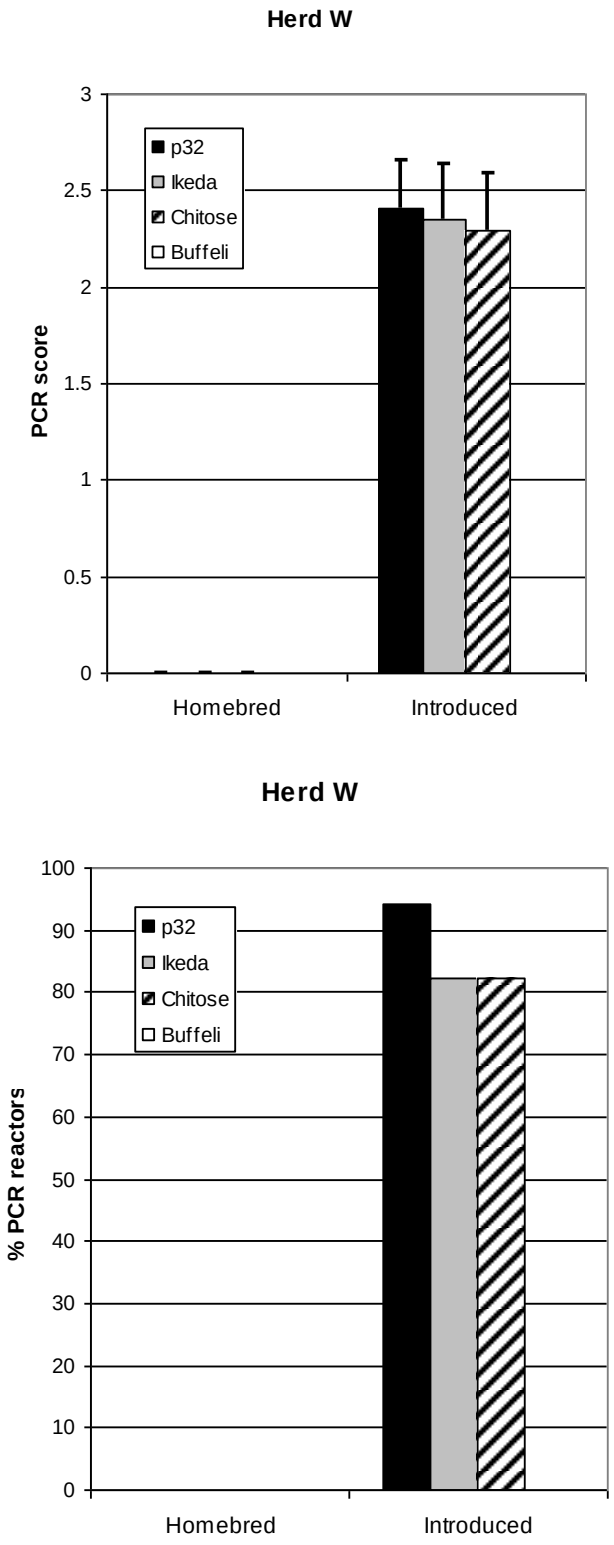


Figure 2. PCR reactivity in Herd W (Tablelands). This represents a single sampling of 10 homebred and 17 introduced adult cows one month after clinical evidence of disease in the herd.

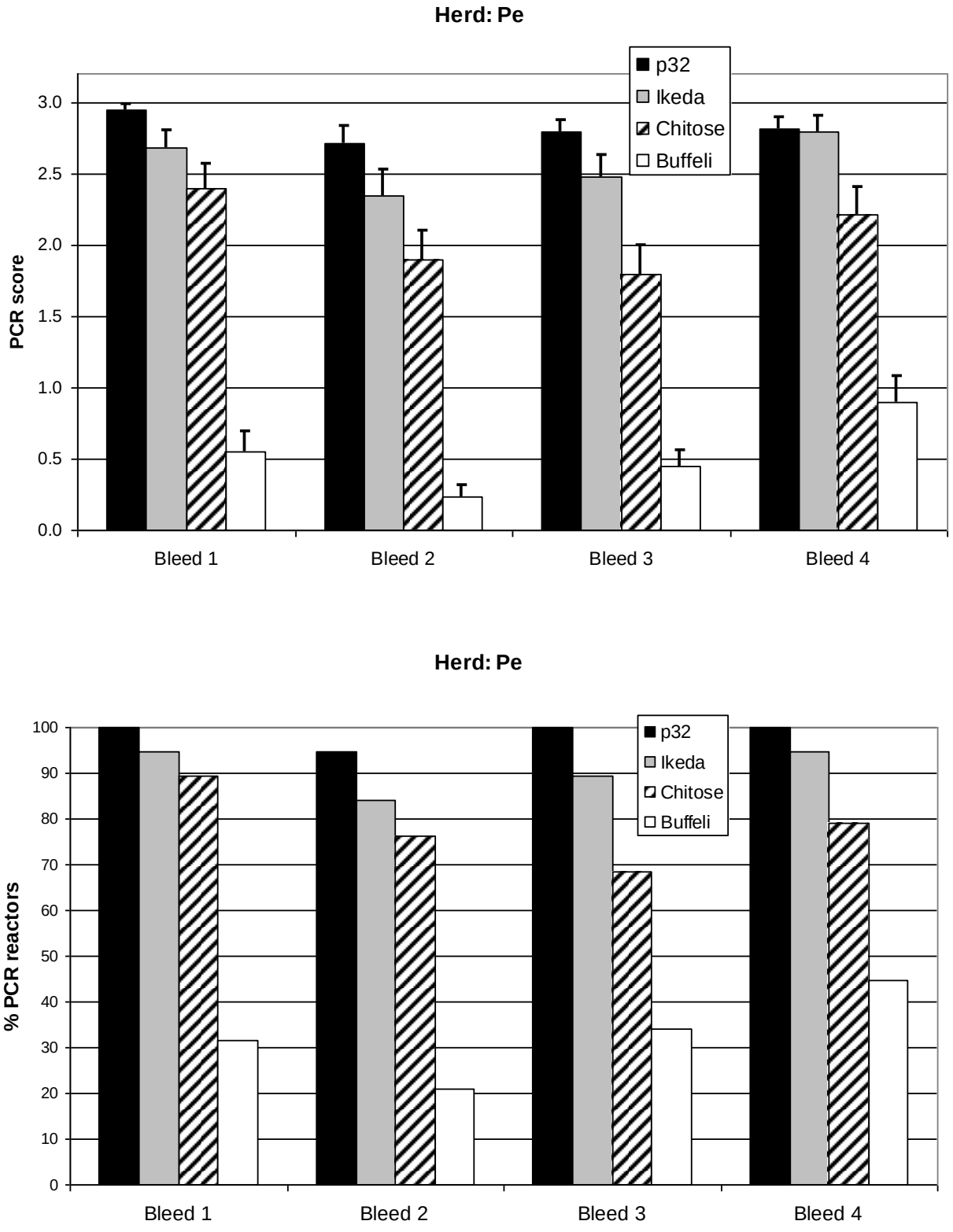


Figure 3. PCR reactivity in Herd Pe (Mid-Coast) over 4 consecutive bleeds 3-4 weeks apart in 38 cattle recently introduced from the Northern Tablelands to the Coast, including 13 born on the Pe property.

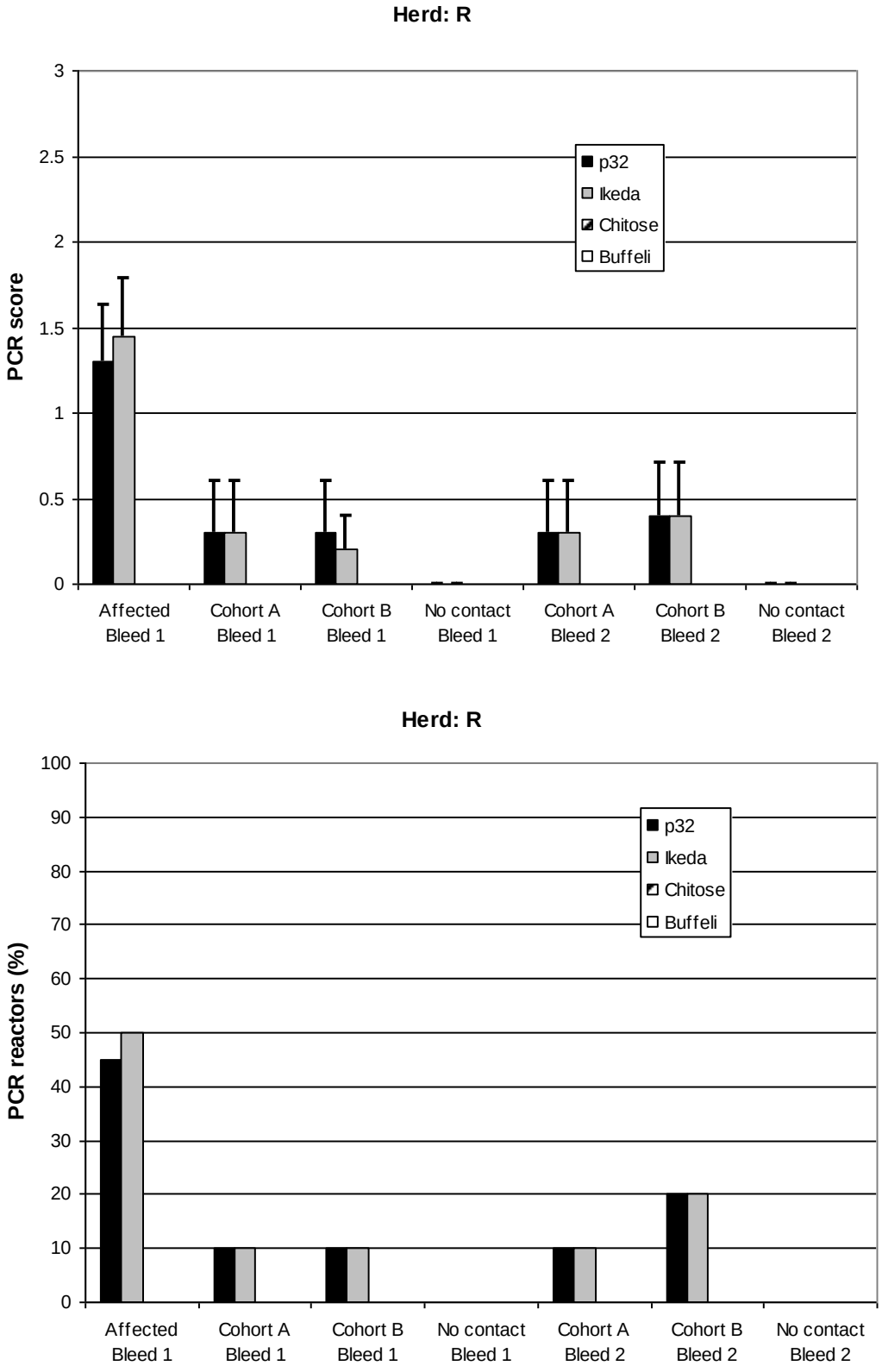


Figure 4. PCR reactivity in Herd R (Hume) in a known affected mob (n =20), and over consecutive bleeds of in two cohort mobs A and B (n = 10, 10) and a third mob (n = 10) with no prior contact with the affected group. Bleed interval is 1 month. Cohort A and Cohort B comprised cattle that were from same 2009 drop as the affected mob, whereas the 'No contact' mob were born in 2008 and were kept separate from all 2009-born cattle.

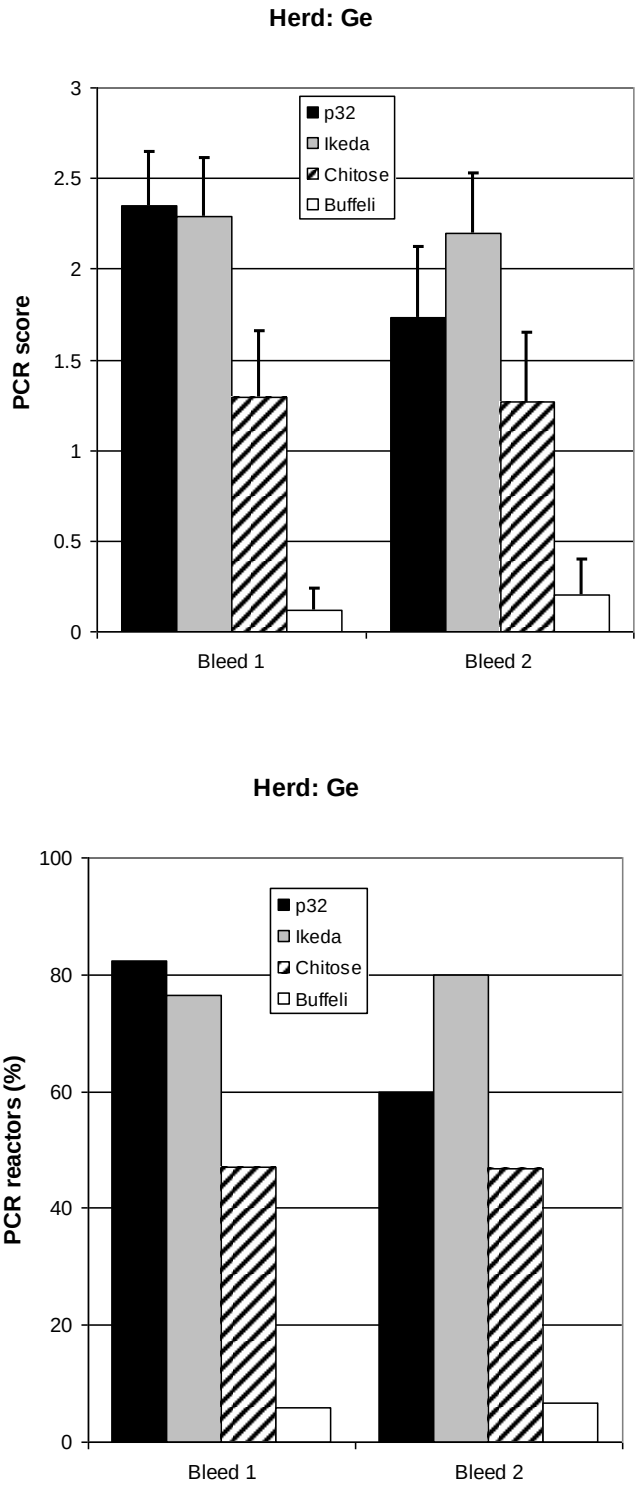


Figure 5. PCR reactivity in Herd Ge (Lachlan), representing consecutive bleeds of 15 cattle 1 month apart.

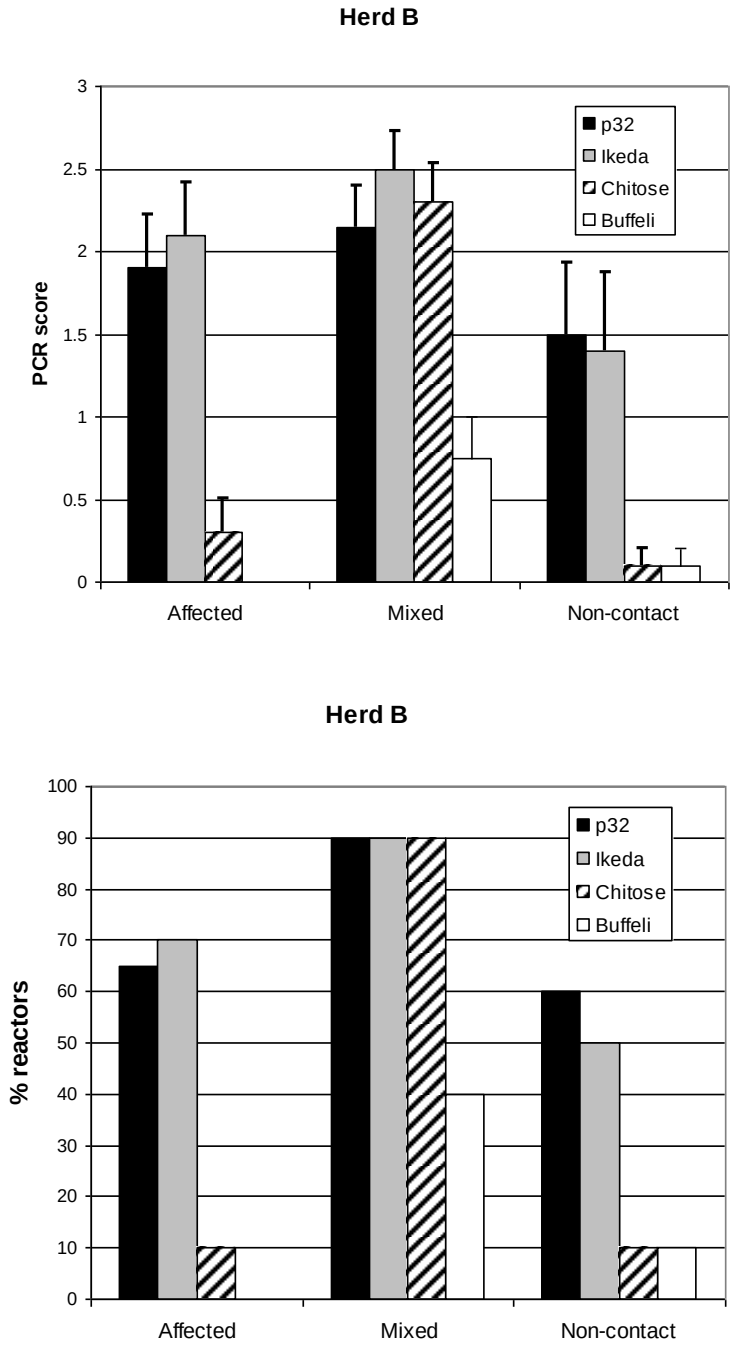


Figure 6. PCR reactivity in Herd B (Hume). In this herd, cattle were retained in either a group whose members had previously been affected with clinical theileriosis (Affected, n =20), a purchased group currently mixed with introduced weaners (Mixed, n = 20), or a group which had no prior contact with affected cattle (Non-contact, n = 10).

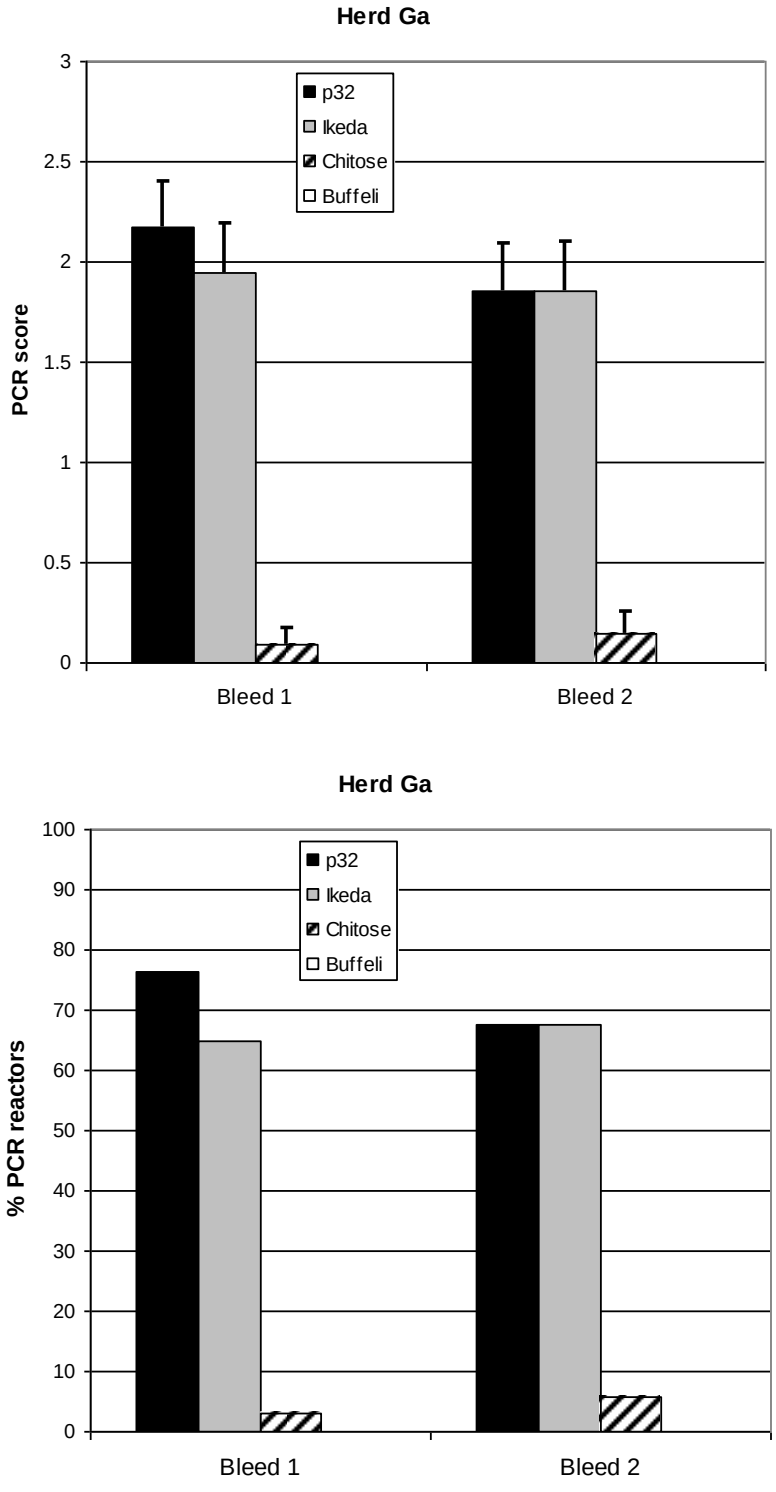


Figure 7. PCR reactivity in herd Ga (Hume), with two consecutive bleeds 1 month apart, the first taken 7 months after evidence of clinical disease.

4.4 Significance study clinical findings and clinical pathology results

The results of clinical and clinical pathology findings are shown in graphic form across the 7 study herds in Figures 8-15. These show the proportion of cattle in each herd with normal or high temperatures (below and above 40^{o}C), abnormalities in mucous membrane colour (with evidence of anaemia by pallor of mucous membranes, or jaundice by yellowing of the mucosae) and abnormalities in erythrocyte appearance. To reduce complexity, the severity of erythrocyte changes (e.g. mild, moderate, severe) for each characteristic in the blood film examinations was not assessed in this report. Many normal cattle show a degree of anisocytosis which was generally reflected across the herds.

Herd Pa animals included calves bled on four occasions and young adult cows and their calves bled once. In the calves subjected to repeated bleeds, theilerias were detected in smears of 56% of calves at the 1st bleed and 100% by the second, and increasing theilerial loads were detected over time (Figure 8). However by bleed 4, only 60% of animals were detected as smear positive (Figure 8).

Young adult cattle in herd Pa were all smear negative whereas their calves within 1 month of birth showed over 90% were smear positive but none were classified anaemic on clinical or haematological grounds (Figure 9). The calves of these cattle were reportedly yarded for some time in hot weather and most revealed high body temperatures (Figure 9); thus it is unclear whether these increased temperatures were due to environmental conditions or disease.

Herd W contained homebred and introduced cattle with generally normal temperatures but the introduced cattle showed distinct differences in mucous membranes, in the proportion of cattle with inclusion bodies in erythrocytes, and more cattle with low PCVs and theilerial forms within erythrocytes (Figure 10). In the introduced cattle, over 10% were anaemic and 65% contained intra-erythrocytic theilerias, with almost 60% having ≥1% of red cells infected (Figure 10). In contrast, only one homebred cow had theilerial forms on smear examination, which were estimated to be in low numbers.

Herd Pe cattle were generally clinically normal and few animals showed elevated temperatures, abnormal erythrocytes on smears or evidence of anaemia on PCV over 4 bleeds (Figure 11). Smears detected an increasing prevalence of theilerias, with prevalence increasing from <15% to almost 90% by Bleed 3, with few cattle (<10%) having ≥ 1% of red blood cells infected at any examination (Figure 11).

In herd R, complete clinical and clinical pathology data was not available from all subgroups. Affected cattle were examined only by PCV and for theilerial organisms on smears, and the latter were not quantified numerically, only recorded as low, medium or high. To enable comparison between subgroups, these terms were adjusted such that “low” were given a score of <1%, “medium” a score of 1-5% and “high” a score of 6-10% when recorded in Figure 12. These are likely to be conservative estimates of the frequency of theilerias in the smears of the affected cattle, but Figure 12 highlights the higher likely frequency in this group compared to other subgroups. Most affected animals had normal PCVs but none of the cohorts to the affected mob had ≥1% erythrocytes with theilerias (Figure 12). Pallor of the mucous membranes and a low prevalence of fever were found in both cohort and non-contact cattle but none showed abnormal PCVs. Smears of non-contact cattle indicated that 10% showed some evidence of theilerial forms at bleed 2, compared with 10-20% of cohort cattle at either of their two bleeds (Figure 12).

Herd Ge, in which cattle were bled on two occasions, showed evidence of mucosal pallor in many animals and 20-40% had marginal or low PCV values at either bleed (Figure 13). Smear results confirmed relatively high frequency of theilerias in erythrocytes at both bleeds, with theilerias evident in >5% of red cells in 15-20% of cattle (Figure 13). Abnormally high rates of erythrocyte abnormalities such as polychromasia, inclusion bodies and poikilocyosis (affecting >30% of cattle) were also evident at one or both bleeds (Figure 12).

Herd B contained three groups: an affected group of 20 cattle, a mixed group of 20 purchased cattle with prior contact with introduced weaners derived from the affected cattle but previously agisted on a coastal property at Taree, and a group of 10 without contact with the affected cattle. In this herd the non-contact mob showed no evidence of theilerias on smears, normal temperatures and mucous membranes and normal haemograms (Figure 14). The affected group contained a high proportion (50%) with jaundiced mucous membranes and 40% showed evidence of anaemia on PCV values with over 50% having positive smears for theileria at frequencies of 1-5% of erythrocytes in over 35% (Figure 14). The mixed group showed no evidence of anaemia or inclusion bodies as seen in the affected cattle, with 10% having pale mucous membranes and <20% showing evidence of theilerial forms on blood smears (Figure 14), although almost 10% contained 1-5% of red cells with theilerial forms.

Herd Ga cattle had little evidence of anaemia or erythrocytic abnormalities, but blood smears did reveal >60% of the 34 cattle bled had theilerial forms at the initial bleed and 30% at the subsequent bleed (Figure 15). At least 1% of erythrocytes contained theilerial forms in 25% of cattle at bleed 1 and 10% at bleed 2 (Figure 15), including one animal with >5% of red cells infected.

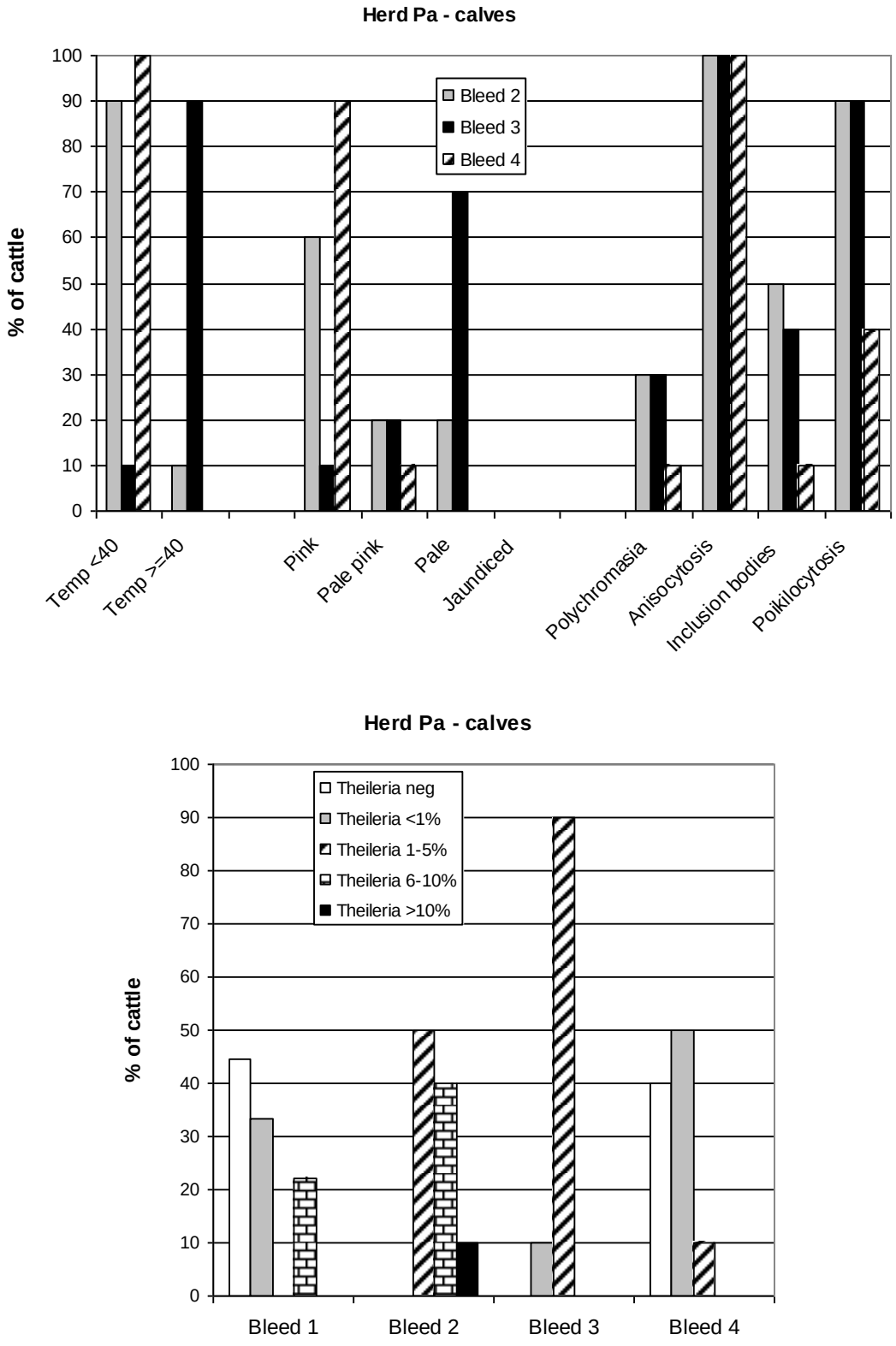


Figure 8. Clinical findings and clinical pathology in calves bled on four occasions in herd Pa. Body temperature and mucous membrane colour were not recorded at Bleed 1.

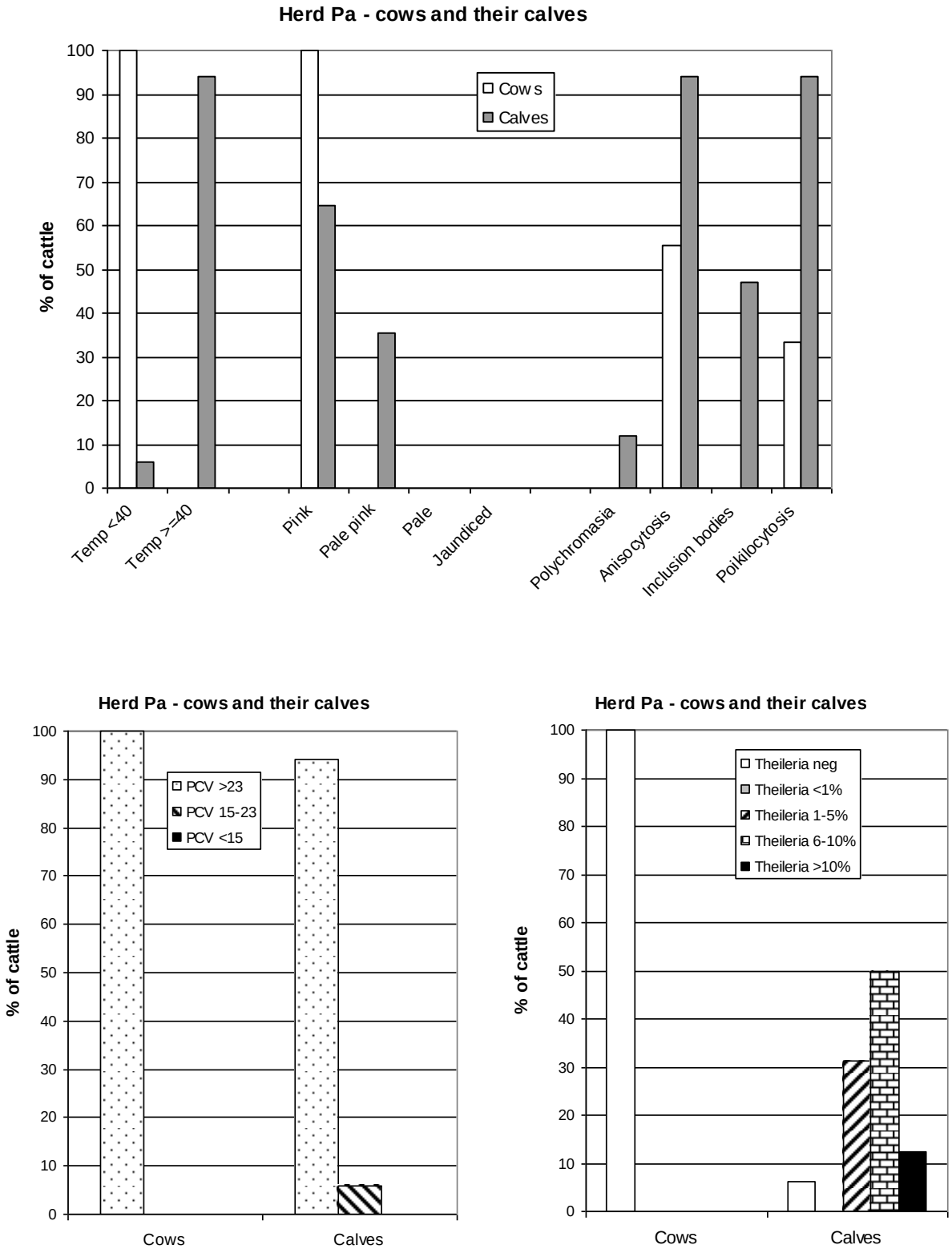


Figure 9. Clinical and clinical pathology findings in 18 herd Pa cows and 17 of their calves, aged < 1 month.

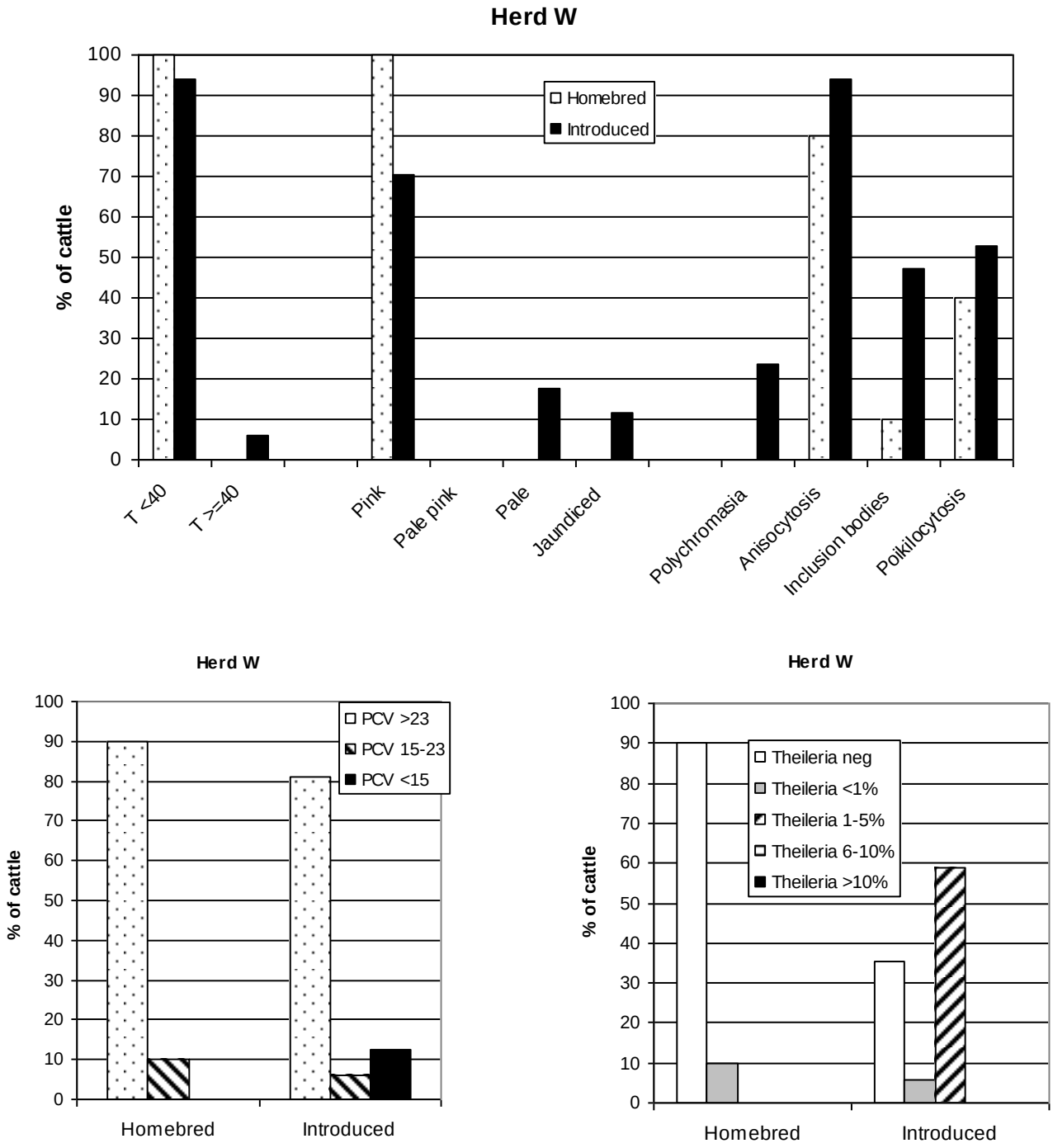


Figure 10. Clinical and clinical pathology findings in Herd W. In this herd a group of homebred cattle (n = 10) are compared with a group of introduced cattle (n = 17).

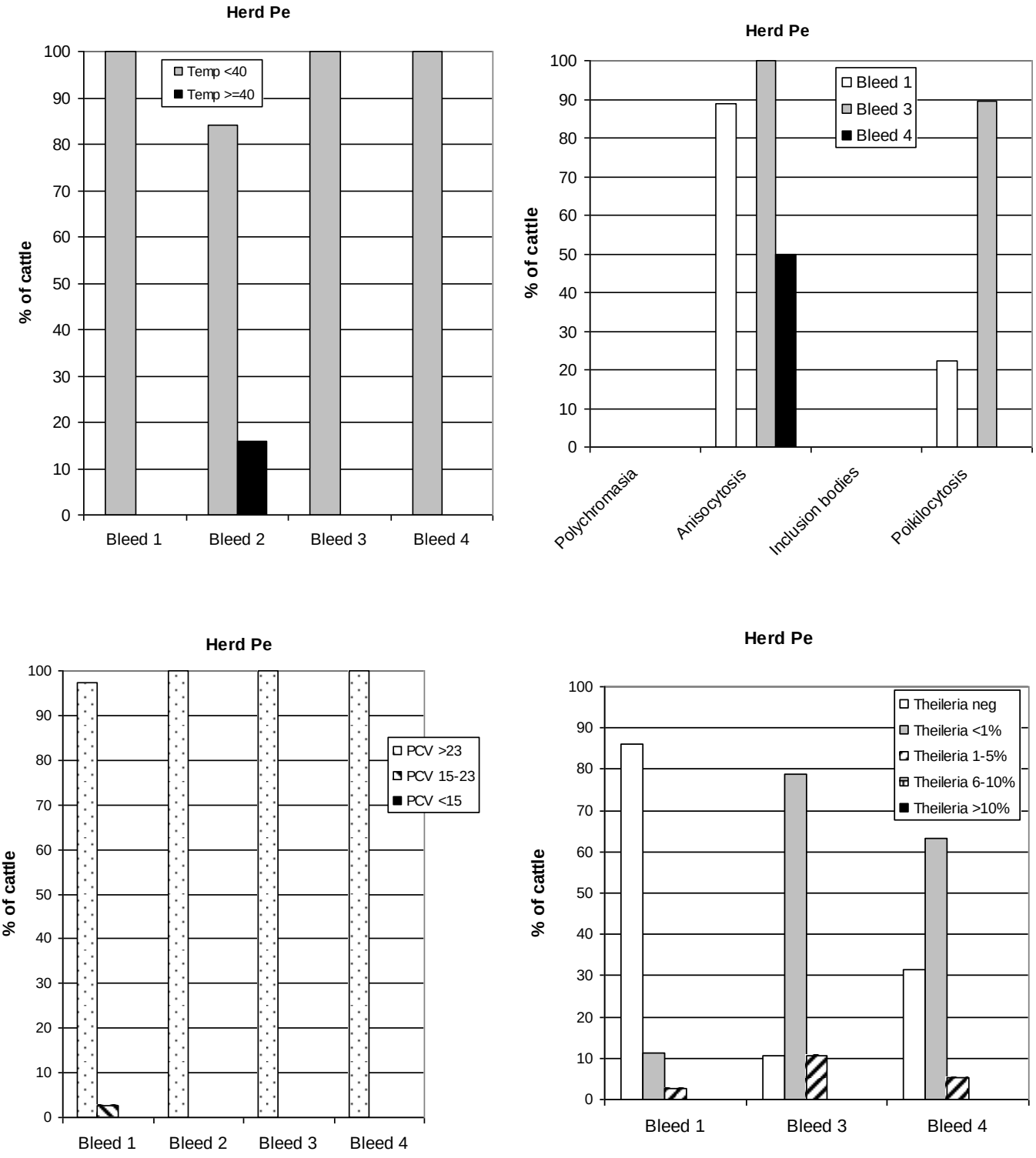


Figure 11. Clinical and clinical pathology findings in Herd Pe, bled on 4 occasions. Smears from bleed 2 were unsuitable for haematological examination.

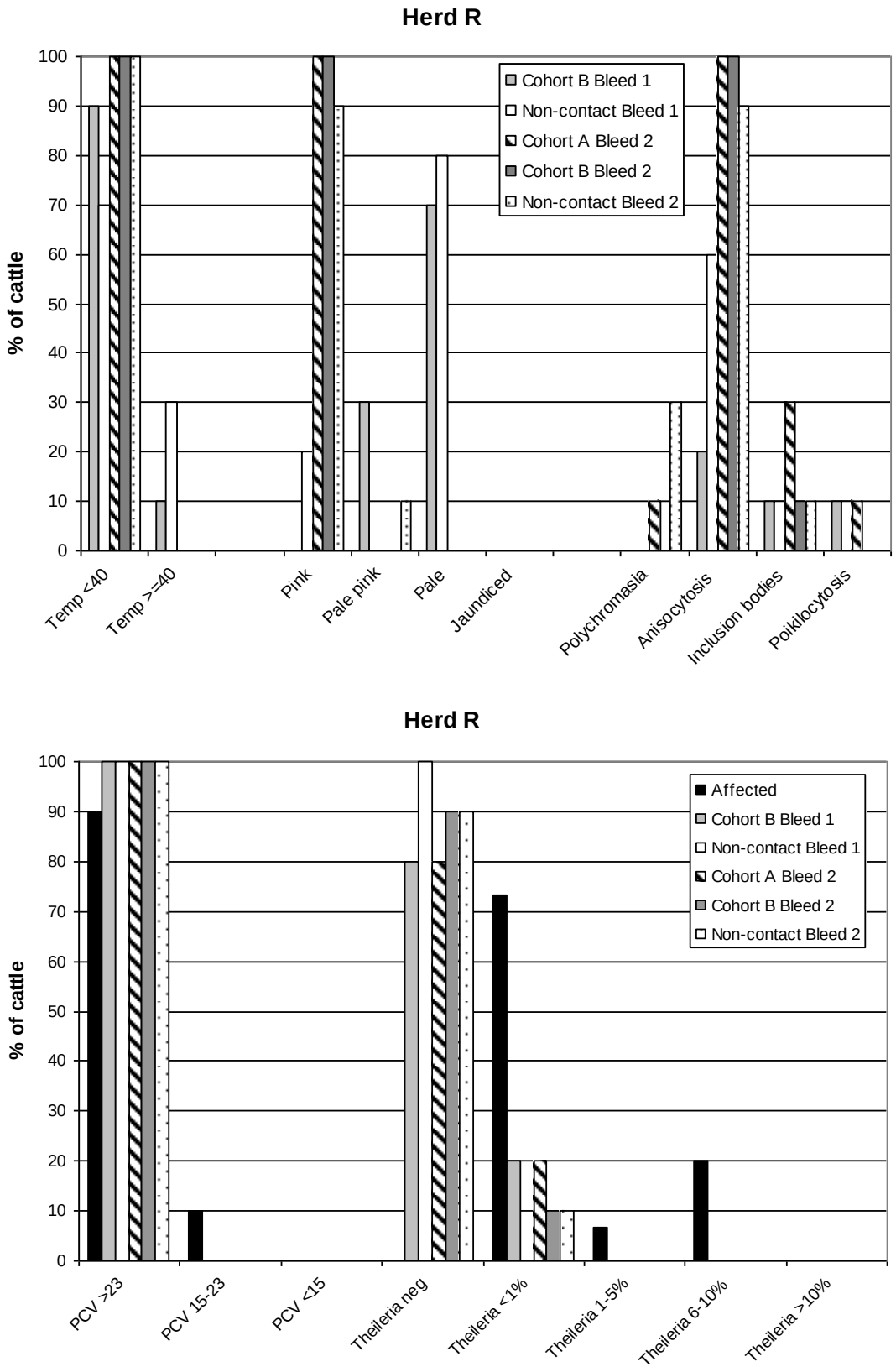


Figure 12. Clinical and clinical pathology findings in Herd R. Affected cattle were only examined for PCV and theilerial-like organisms on smears. Cohort A animals were not examined fully on Bleed 1, so data for this group was excluded.

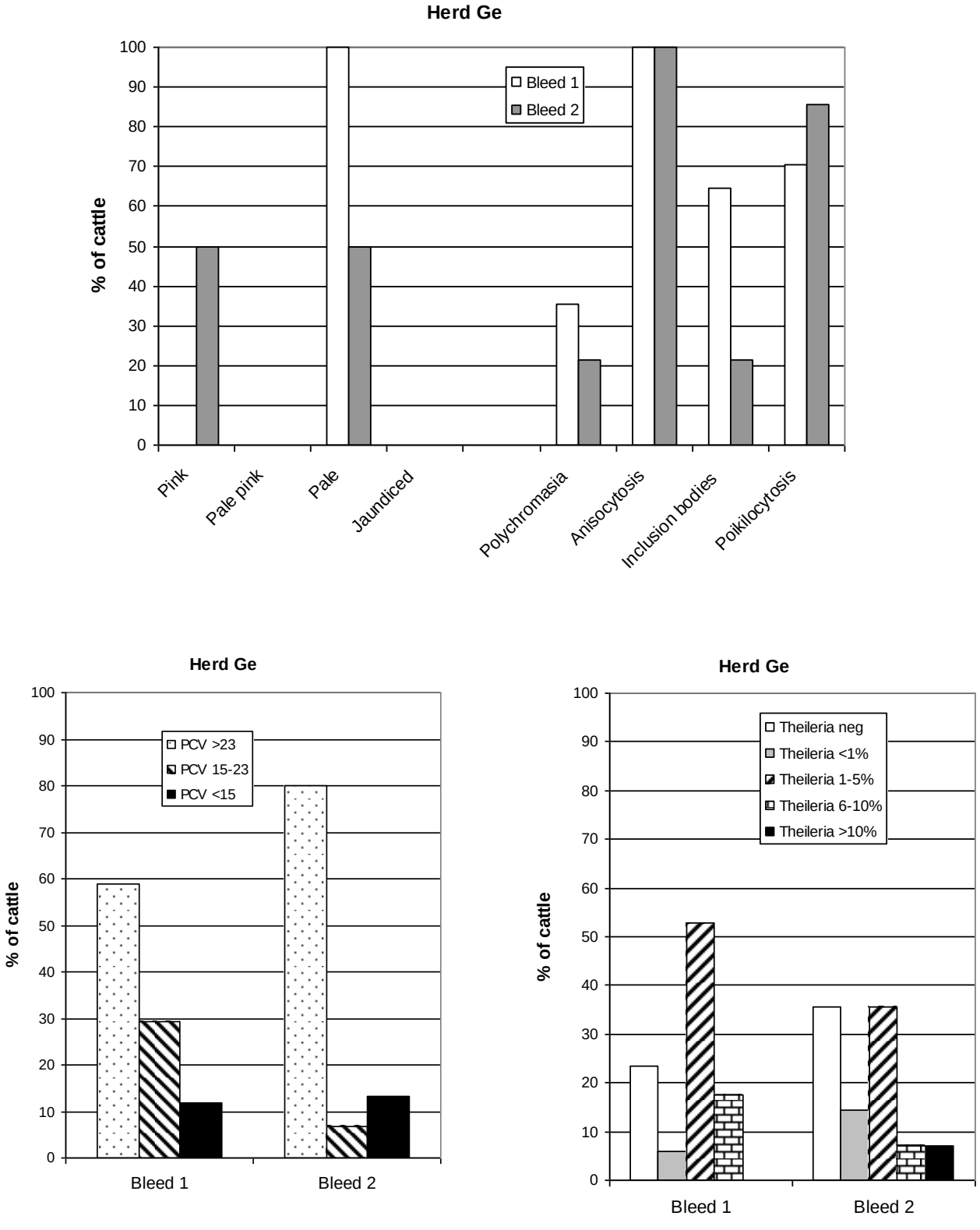


Figure 13. Clinical and clinical pathology findings in herd Ge, bled on two occasions.

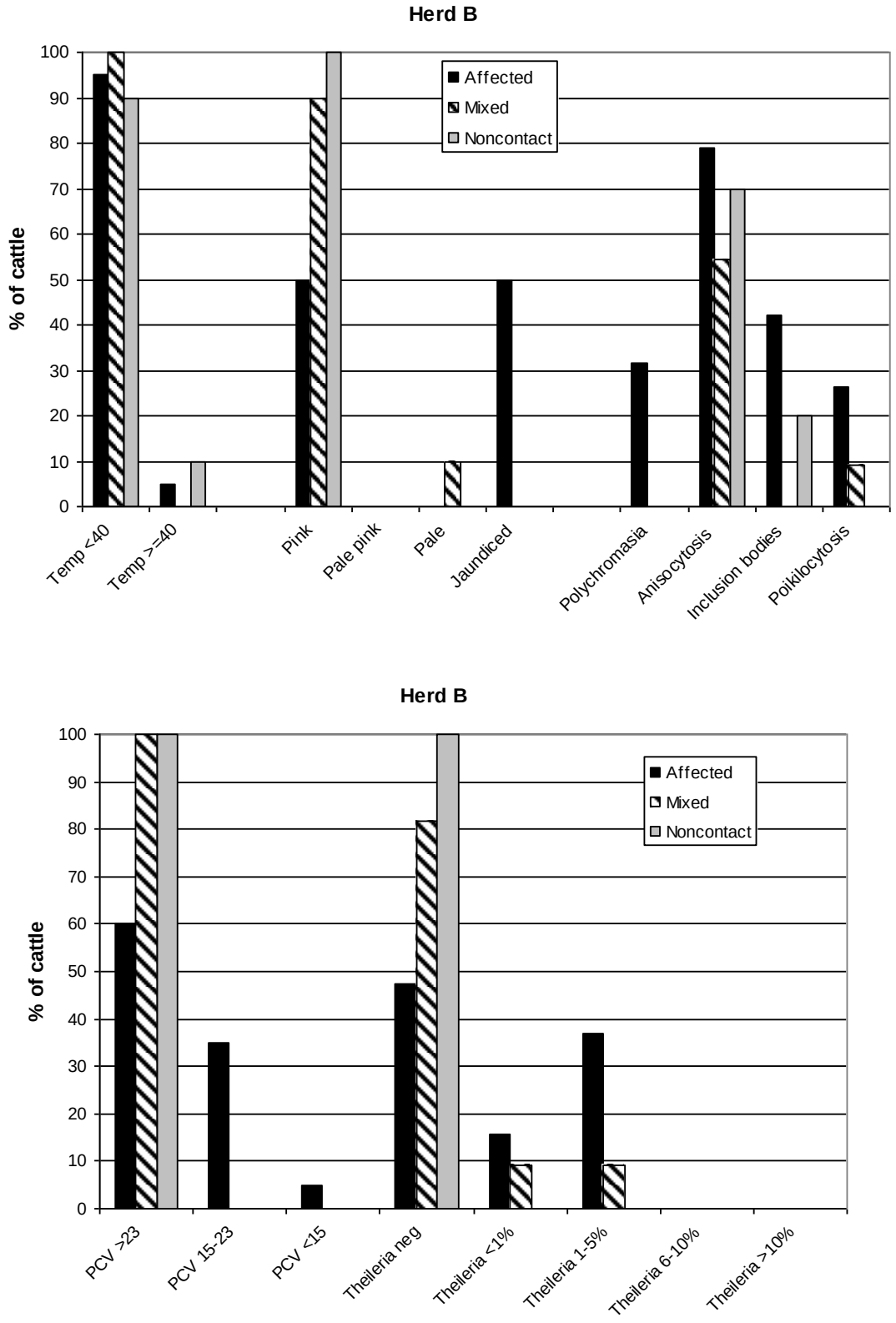


Figure 14. Clinical findings and clinical pathology in herd B, where three separate mobs of cattle were examined: an affected group with prior clinical theileriosis (n =20), a group which contained animals that had been mixed with those in the affected mob (n = 20), and a group that had no contact with the affected mob (n = 10).

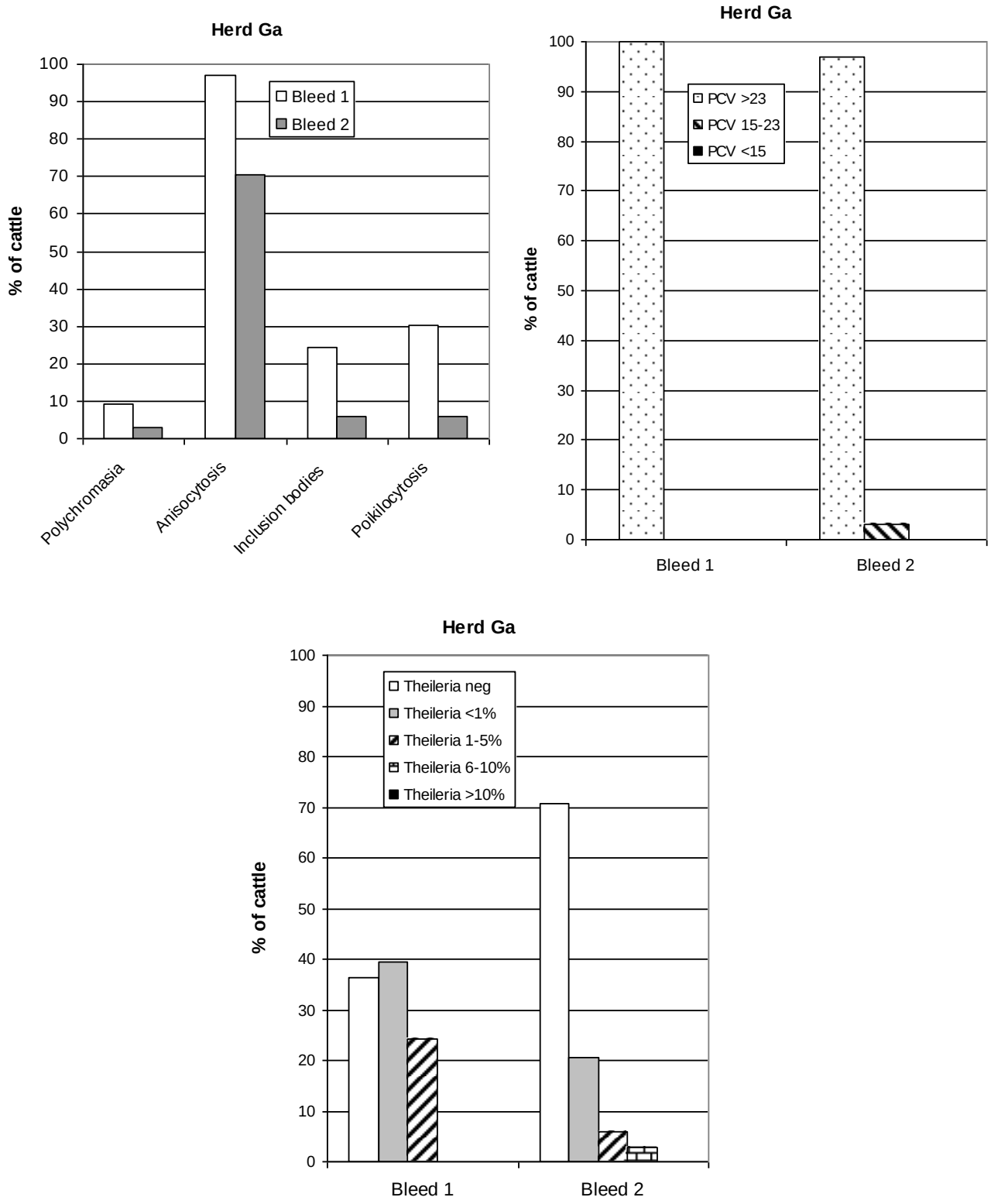


Figure 15. Clinical pathology findings in herd Ga, where 34 cattle were sampled on two occasions.

4.5 Diagnostic implications of significance study findings

The overall PCR findings and smear findings across all 484 samples collected in the significance study were further analysed by examining the associations between results of smears and PCR assays. Table 16 and Figures 16-21 depict the results of these analyses. When comparing the p32 screening PCR assay with subsequent MPSP type specific assays, there was a highly significant association between the results of these assays ($P < 0.001$) and a low rate of false negative results (2.3%) for the p32 assay (Table 16).

Table 16. Association between screening PCR (p32) assay and MPSP type PCR results among 484 samples

	p32 PCR positive samples	p32 PCR negative samples
MPSP PCR positive[1]	342 (71%)	18 (3.7%)
MPSP PCR negative	11 (2.3%)	113 (23%)
Chi squared value	348.01	
P value	<0.001	
Correlation coefficient (r)	0.85	

[1] PCR result positive for Ikeda, Chitose or Buffeli

While 90% of smear positive samples coincided with PCR positive results, over half of the smear negative samples were also PCR positive (Figure 16). The majority of the smear positive, PCR negative samples were in samples categorised as containing theilerial forms in <1% of erythrocytes, whereas almost all samples with 1% or more affected erythrocytes were PCR positive (Figure 17).

The presence of single and multiple MPSP types among samples with or without smear evidence of theileriosis is described in Figure 18. In 218 smear positive samples from the seven affected herds in the significance study, Ikeda type predominated. This was the sole MPSP type detected in 35% of smear positive samplings, compared with 0 and 2% of sole MPSP detections of Buffeli and Chitose types respectively. The combination of Ikeda and Chitose types only was found in a further 37% of smear positive samples, and all three MPSP types were detected in an additional 14% of the smear positive samples (Figure 18).

An assessment of the value of parallel testing of two DNA extracts (one neat, one at a dilution of 1:10) is shown in Figures 19 and 20. The use of diluted DNA extract enabled detection of 35% of all samples in the p32 PCR and 25% of samples in the Ikeda PCR (Figure 19). The use of diluted extract equated to the detection of 49% of the p32 PCR positive samples, and 33%, 31% and 52% of the PCR positive samples in the Ikeda, Chitose and Buffeli assays respectively (Figure 20). In the p32, Ikeda, Chitose and Buffeli assays, the use of only one extract would have detected 51%, 67%, 71% and 49% respectively of the positives for the neat extract alone compared with 93%, 87%, 88% and 83% respectively for the diluted DNA extract alone. For blood samples classified as theileria smear positive and negative, similar analyses are presented in Figure 21. A greater proportion of smear negative samples tested PCR positive at the 1:10 dilution of DNA extract than smear positive samples, regardless of the PCR assay (Figure 21).

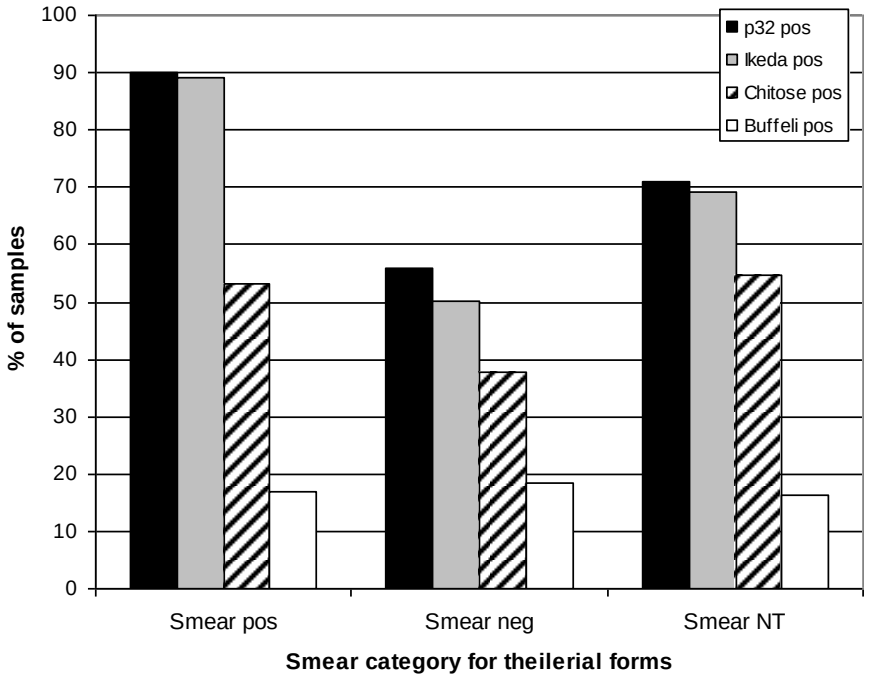


Figure 16. Proportion of 484 samples testing PCR positive in four assays among different categories according to detection of theilerial forms on smears. Smears are categorised as smear positive (pos; n = 218), smear negative (neg; n = 211) or not tested/unsuitable for smear examination (NT; n = 55).

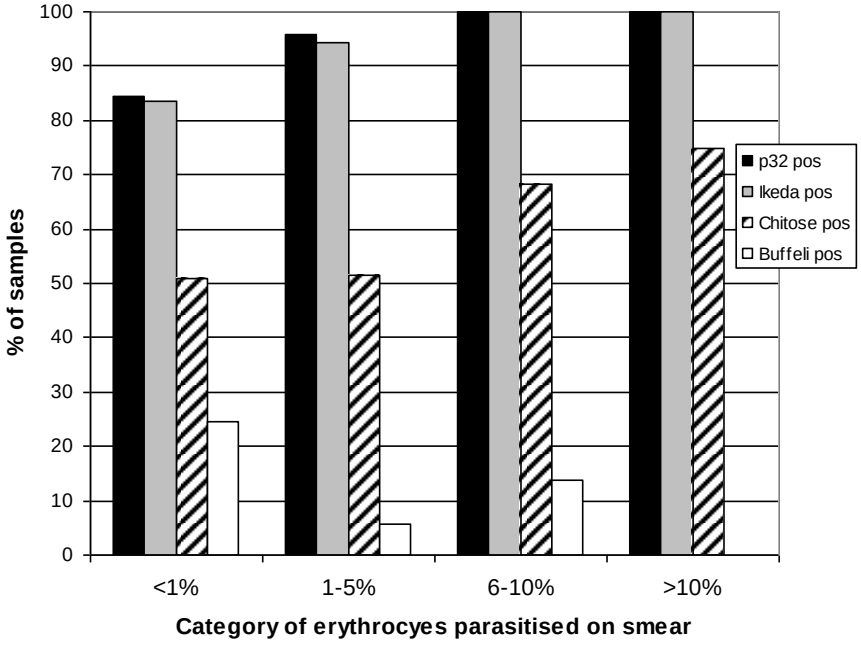


Figure 17. Proportion of smear positive samples testing PCR positive among different levels of parasitaemia as judged by smear examination. In each category, numbers of samples were: <1% n = 122; 1-5% n = 70; 6-10% n = 22; >10% n = 4.

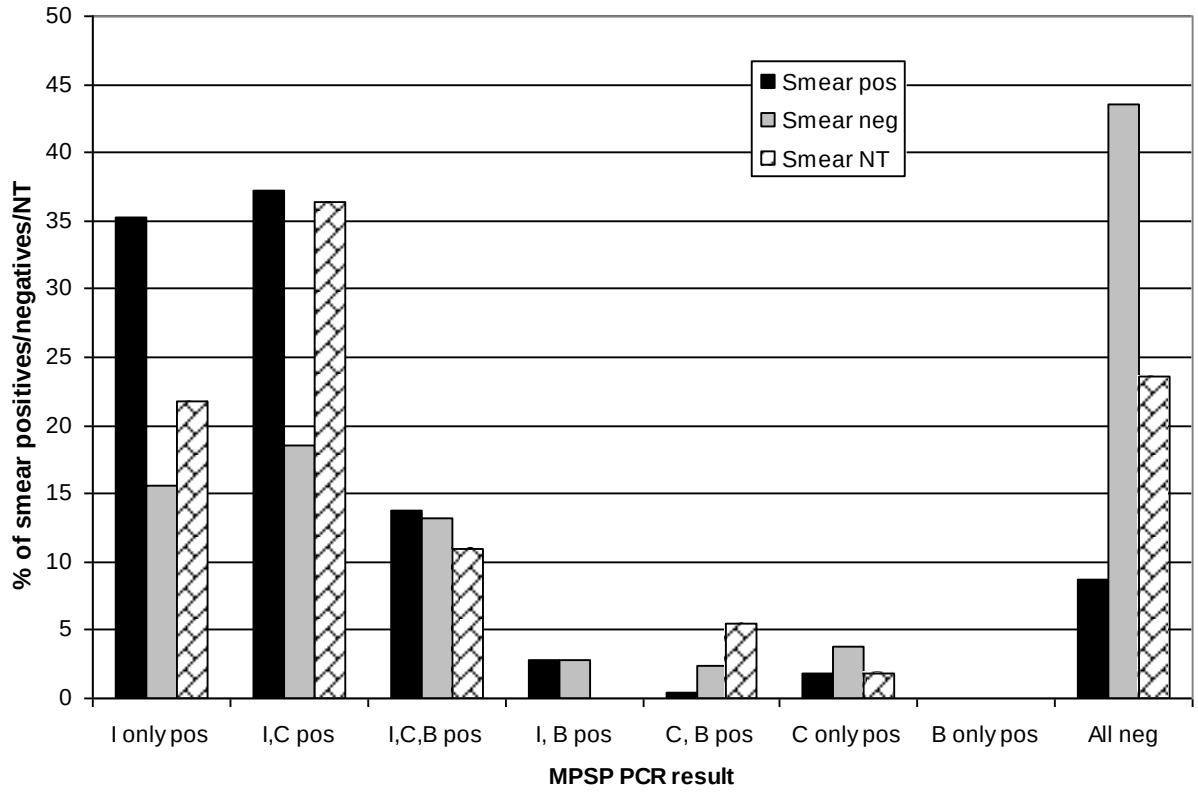


Figure 18. Proportion of samples that were positive in one or multiple PCR assays for Ikeda (I), Chitose (C) and Buffeli (B) when compared with evidence of theilerias on smears (Smear pos), no evidence of theilerias on smears (Smear neg) or in samples where smears were unsuitable and not tested (Smear NT).

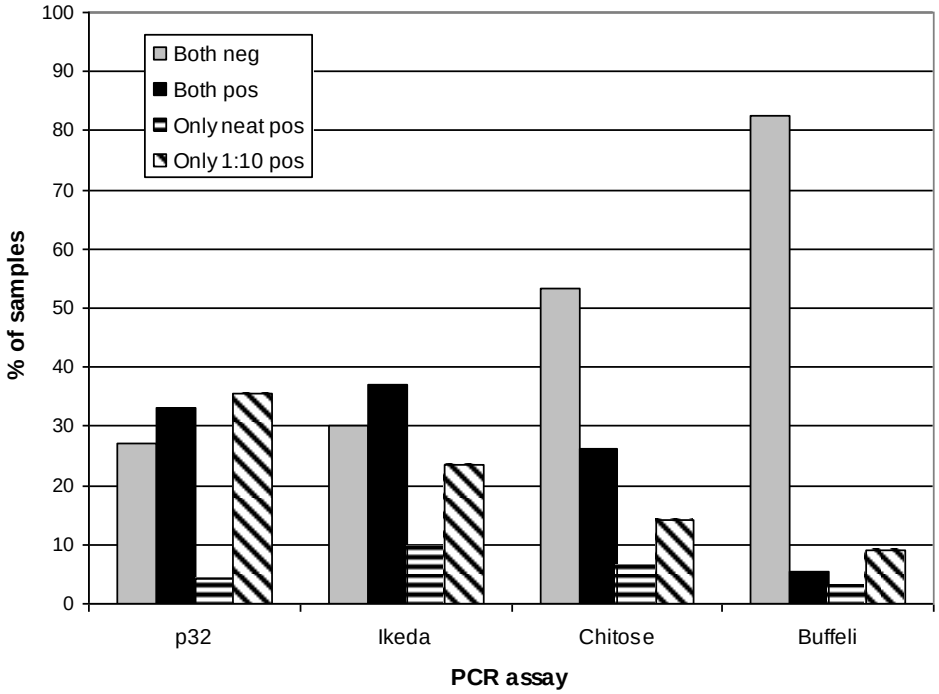


Figure 19. Proportion of 484 samples that yielded concordant or discordant results from DNA extracts tested neat and at a 1:10 dilution. Striped bars indicate proportion of samples undetected if that extract was not tested in parallel.

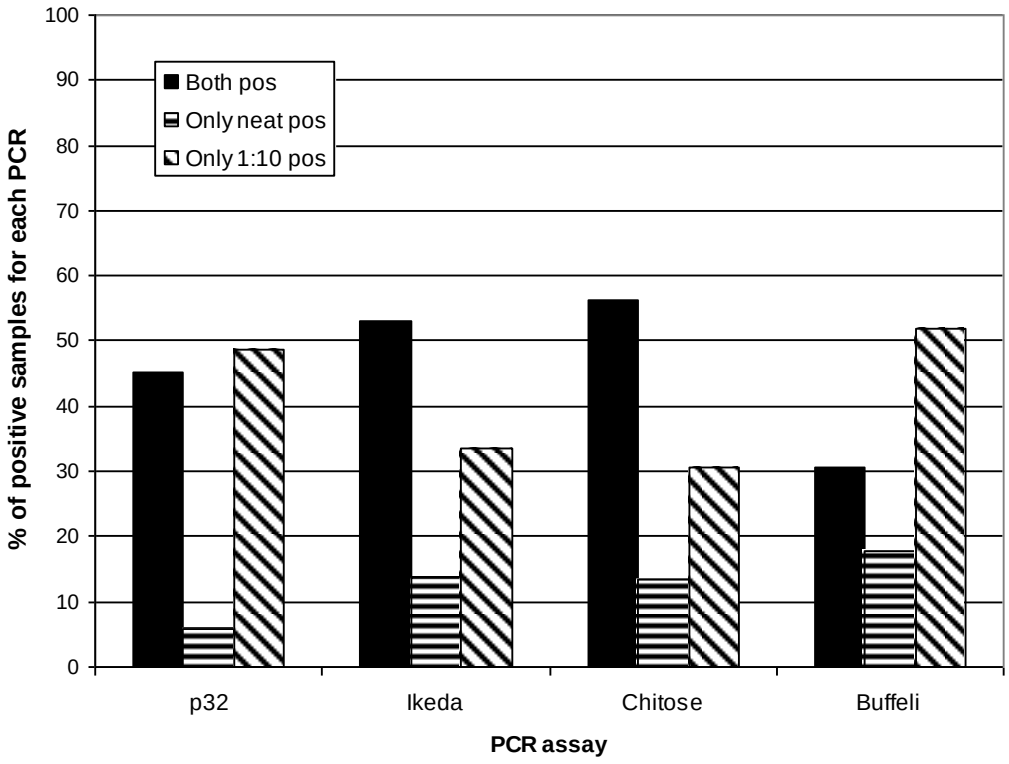


Figure 20. Proportion of PCR positive samples for each PCR assay that were positive in tests of two DNA extracts (neat and 1:10), or only positive in one extract. The total positive samples for each PCR were: p32, n = 353; Ikeda, n = 338; Chitose, n = 226; Buffeli, n = 85.

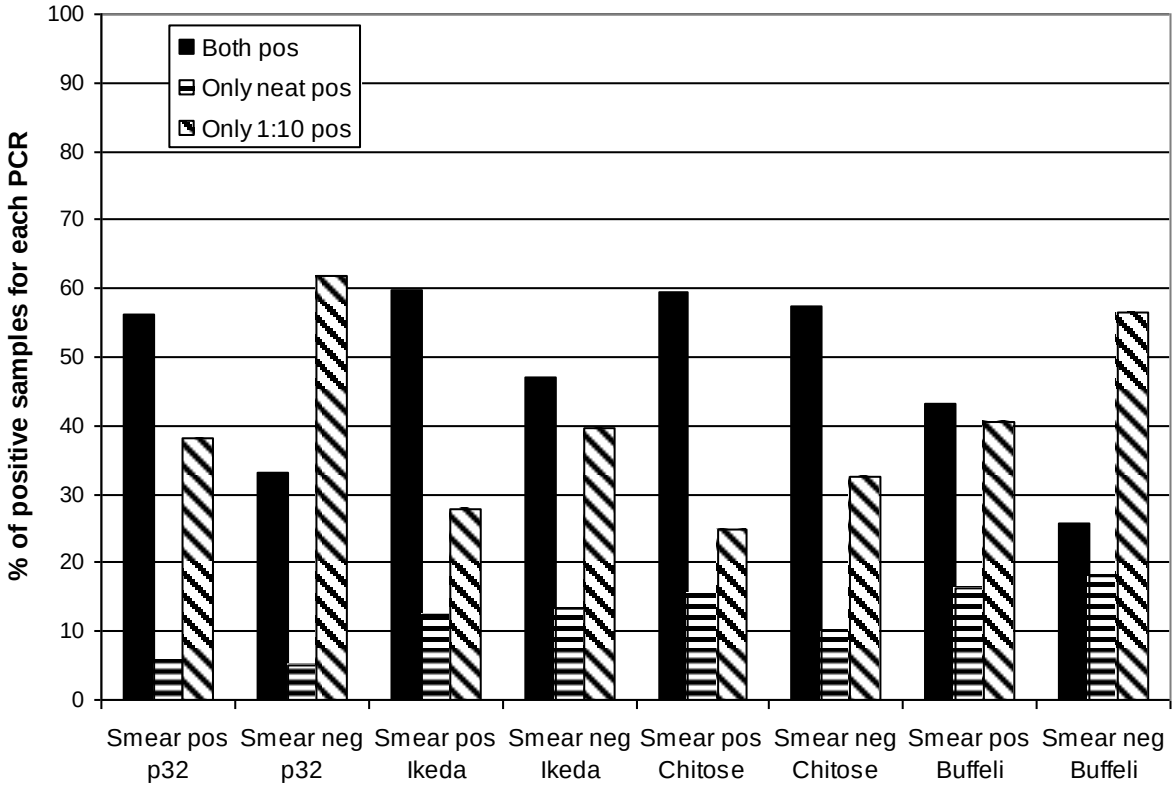


Figure 21. Proportion of PCR positive samples for each PCR assay that were positive in tests of two DNA extracts (neat and 1:10), or only positive in one extract, based on blood samples categorised as smear positive or negative. The total smear positive/negative samples that were PCR positive for individual assays was: p32, n = 196/118; Ikeda, n = 194/106; Chitose, n = 116/80; Buffeli, n = 37/39.

5 Discussion

5.1 Distribution study

Based on studies such as Zakimi *et al* (2006), conventional PCRs for various MPSP genes have been described. The PCR assays that target the p32 (common) MPSP gene sequence and the specific MPSP sequences for the Ikeda, Chitose and Buffeli types as described by Zakimi et al (2006) were modified in the use of PCR cocktail reagents that are readily available in Australia, and increased template volume, based on developmental studies by our group at the Elizabeth Macarthur Agricultural Institute (EMAI) in 2009-2010. The sequences of the primers used are as described by Zakimi et al, and include additional nucleotides at their terminal end encoding a restriction enzyme cutting site. These additional nucleotides do not match the GenBank sequence of the MPSP genes targeted. As restriction enzyme analysis (REA) is not part of the current procedures, these extra nucleotides are not utilised.

The p32 gene sequence targeted in these PCR procedures encompasses the gene sequence of one of the three specific MPSP types Ikeda, Chitose or Buffeli. This is advantageous as it also targets a common sequence that enables a common reverse primer to be used for all four PCR assays, thus reducing the number of primers required.

Blood samples, the key source of theilerial DNA in this study, may contain PCR inhibitors and produce false negative PCR results. Additionally, if samples contain excessive target DNA, the PCR reaction may be unbalanced and also give false negative PCR results. In order to overcome these issues, our earlier studies in testing DNA extracts using the DNeasy system have indicated that testing is more reliable if DNA extracts are tested using both neat extract and a 1:10 dilution. If either extract gives a positive result, the sample is considered PCR positive. In addition, our earlier work confirmed that a PCR positive result in any of the MPSP-specific assays (Ikeda, Chitose or Buffeli) correlated very highly with a positive result in the p32 assay. For this reason, the distribution study was able to be streamlined by screening samples with the p32 assay and then only testing those samples with a positive result in the MPSP-specific assays.

As a result of the distribution study, we were able to confirm that the Victorian herds sampled (and essentially those from East Gippsland) commonly contained a number of cattle with Chitose or Buffeli infections, and these were present in approximately 25% of samples (Tables 12, 13). However, Ikeda infection was rare in these Victorian herds, and confined to a single herd, with only one animal of 10 testing positive for this type. The Queensland herds sampled showed a similar prevalence of Buffeli type (25% of samples), and this type was widespread (4/5 districts) and evident in 14/20 herds (Tables 9, 10). Queensland herds contained a higher prevalence of Chitose type (44% of samples) than Victorian herds, and this type was also widespread (4/5 regions) and very frequent (47-70%) in some herds and regions (Table 10). The Ikeda type was also detected in 3/5 regions, but at a lower prevalence (11%) than the other two types (Table 9), and a slightly higher prevalence among herds located in the South-East and North regions (Table 10).

In NSW, Buffeli and Chitose infections were mainly prevalent in the North Coast and North West regions, with few occurrences in other regions. The statewide prevalence was estimated at 11-19% for these two MPSP types, but these two regions showed much higher prevalences (44-65% for Chitose, 19-47% for Buffeli). In fact, Buffeli type was not detected outside these two regions. The Ikeda type, unlike Queensland, was only detected in cattle in one region; the North Coast herds harboured this type with a prevalence of 74%, and this type was detected in all 4 herds sampled in that region. These findings suggest that Ikeda, despite its association with

clinical cases in cattle in several regions of NSW, is not readily detected in herds where the risk of theileriosis is low, or in regions with little clinical history of disease. Further, since the Buffeli strain was not detected outside the North Coast and North West regions of the state, and Chitose was also mainly confined to these regions, then it is possible that a similar vector may be associated with the movement of all three MPSP types in NSW.

The virulence of the strains of Ikeda or other MPSP types detected is not known, nor if there are genetic differences (variant DNA profiles) within isolates of a particular MPSP type. It is therefore necessary to establish whether the strains detected in herds where disease has not been reported are different from those where clinical disease has occurred.

5.2 Significance study

The significance study investigated several NSW herds with a known history of clinical theileriosis, and some were located in regions of NSW where PCR testing of cattle herds suggested (based on the distribution study findings) that homebred cattle were unlikely to be normally infected with MPSP types Ikeda and Buffeli, and Chitose type infection was also likely to be uncommon (Table 12). All herds were shown to contain animals infected with Ikeda type, while 5/7 had high carrier rates of Chitose type organisms and the remaining herds had either low (herd Ga) or no evidence (herd R) of this type. Only three herds showed moderate prevalence of Buffeli type strains (herds Pa, Pe, B), one showed low prevalence of this MPSP type (herd Ge) and the remaining three (herds W, R, Ga) had no evidence of Buffeli infection. These results indicate Ikeda is the most important MPSP type associated with clinical theileriosis in NSW, and is in agreement with our studies of clinical theileriosis cases investigated at EMAI since 2010. The importance of the Ikeda type is highlighted in the analysis of samples that were PCR positive in any assay (Figure 18), where the Ikeda type was identified in the absence of other MPSP types from 35% of smear positive blood samples, and the prevalence of the Ikeda type was almost identical to that of the screen test (p32) PCR among smear positive samples regardless of the category of parasitaemia from <1% to 10% (Figure 17).

Homebred cattle in herd W indicated these were free of benign theileriosis on PCR testing (Figure 2), as were animals without contact with a known infected group in herd R (Figure 4). However in herd B, where a clinically-affected mob had an estimated 70% carrier rate of Ikeda type organisms, 50% of cattle without direct contact with this mob were found to be harbouring Ikeda type organisms (Figure 6). The mobs in herd B were set stocked, with no boxing of different groups in the same yards, nor movement of one group onto paddocks previously occupied by another group. However, at some stages, fence contact between groups was possible. Thus spread within herds was variable at the time point selected for sampling.

Young calves appear to be high prevalence carriers of benign theilerias, as seen in herd Pa where calves at 1-2 weeks demonstrated carrier rates of 50-60% for Ikeda and Chitose strains, and within one month this prevalence rate had increased to 100% based on PCR testing (Figure 1), where it remained relatively stable for at least a further two months. In the same herd, pregnant heifers with prevalence rates of 90% benign theilerias, were shown to produce calves with identical prevalence rates when the calves were tested at less than one month of age (Figure 1). These calves also had higher prevalence rates for Ikeda strains than their dams. Of particular interest were the differential prevalence rates over time for the MPSP types, where Buffeli type slowly increased whereas Ikeda types increased rapidly and Chitose type prevalence increased moderately. It is possible that Ikeda strains in this herd could outcompete other MPSP types. Whether this occurs within the host or within a vector such as a tick is unclear, but other

workers have suggested a related phenomenon, whereby experimentally infected cattle show loss of Buffeli infection after a few weeks in the presence of Ikeda and Chitose infection (Kamau et al., 2011a). These workers suggested that co-infection may cause an interaction and possible interference between MPSP types, that may lead to loss of one or more types. Since studies with *Theileria parva* have shown a less virulent strain has a longer incubation period and is slower to reach high levels of parasitaemia (Tindih et al., 2010), a similar situation may apply when co-infection occurs with Ikeda, Chitose and Buffeli types.

In herds introducing cattle back to the Coast from the Northern Tablelands, such as herd Pe, introduced stock may show a very high prevalence rate of benign theileriosis infection at testing a few weeks after introduction. This rate then can remain unchanged over the ensuing 3 months, with 90% of cattle harbouring Ikeda and 70-90% harbouring Chitose type organisms (Figure 3).

In two herds where contact with cattle of high risk status occurred (herds B, R) the outcome of testing was dependent on the disease stage when the mixing had occurred. In herd B, where cattle were currently mixed with introduced coastal origin weaners, high prevalence rates of Ikeda and Chitose infection were detected, higher than those of a clinically affected mob on the same farmland (Figure 6). In herd R, where cattle of the same age were kept separately from a clinically affected mob, these cohort animals showed a prevalence of Ikeda infection about half of that of the infected mob at each of two bleeds (Figure 4).

The stability of carrier rates of Ikeda and Chitose types over time is demonstrated in herds Ge and Ga, where very similar proportions of reactors and similar degrees of reactivity (by mean PCR score) were detected for these MPSP types at intervals of 1-7 months. These findings are consistent with long term infections suggested for benign theilerias of the Buffeli type (Callow, 1984).

The clinical and clinical pathology data highlights the value of mucosal membrane colour, PCV and quantification of the proportion of erythrocytes showing theilerial forms in assessing infection severity. Pale and jaundiced mucosae were associated with clinically affected groups of cattle, and herds showing numbers of cattle with ≥1% erythrocytes containing theilerias were associated with high carrier rates of benign theilerias of the Ikeda type, with or without Chitose type. However, overall PCR testing appeared more sensitive than smear examination. For example, high prevalence rates based on PCR were found in groups of adult cattle in herd Pa without clinical evidence or high counts on smears. While herd Pa adults and calves had similar PCR reactivities, only the calves showed high numbers of organisms on smears (Figures 1, 8, 9). Also in some calf bleeds in this herd, PCR was superior in detection of theilerias. At bleed 4, 100% showed PCR positive status for Ikeda, yet smears detected only 60% as positive (Figures 1, 8). Similarly in herd B, more animals were detected as PCR positive in all subgroups (affected, mixed, non-contact) than smears (70 vs 52%, 90 vs 29%, 60 vs 0% respectively)(Figures 6,14). Likewise in herd Ga, where approximately 70% were PCR positive on each of two bleeds, smears detected 29-63% positive only (Figures 7, 15).

In some cases, low level detection of theilerias on smears did not match a positive PCR status. In herd W, homebred cattle were PCR negative but 1/10 indicated low numbers (<1%) of theilerial forms on smears; in contrast in introduced cattle, PCR detected over 90% as positive in the p32 assay (and over 80% in the Ikeda and Chitose assays), yet smears detected <70% of animals as positive (Figures 2, 10). Similarly in herd R, where almost 75% of an affected mob of cattle were reported to contain theilerial forms in smears at low numbers (<1% of erythrocytes),

only 50% of all animals were detected as PCR positive (Figures 4,12); in the same mob only 25% comprised cattle with ≥1% infected erythrocytes. In other herds, the proportion of smear positive and PCR positive cattle matched. In herd Ge, similar proportions were negative in both tests (Figures 5, 13), but these results coincided with a predominance of the smear positive cattle having >1% of erythrocytes visibly infected. These results indicate that either some smears recorded as containing low numbers of theilerias (<1% of infected erythrocytes) are false positive, or that PCR assays may fail to detect some cattle with very low parasitaemias. False positive smears describing low numbers of theilerias may be due to stain artefacts, since the Diff Quick strain can cause deposition of bluish debris resembling theilerial forms. Since intra-erythrocytic theilerias can vary greatly in their morphology, from coccoid forms or small rods to signet ring shapes, smears examinations can be subjective particularly if low numbers of likely forms are detected. Alternatively, if the PCR primers are not an exact match for all strains of benign theilerias in some herds, or if the DNA extraction process was not optimal for some samples, the PCR assay may give a false negative result. Further investigation of such samples, including MPSP sequence studies, is therefore of merit. In addition, modification of the PCR assay to include an internal control to assess the reliability of DNA extraction would be of value.

Over time, the number of cattle with detectable theilerias in smears was also shown to decrease, whereas PCR detection remained high. In herd Pe, smears detected <20%, >90% and <70% cattle with theilerias at three bleeds, while PCR detected >90% on all these occasions (Figures 3, 11). Similarly in herd Ga, smear detection fell from 63% to 29% over two bleeds one month apart, whereas PCR testing indicated a constant detection rate of 65-70% at each bleed (Figures 7, 15).

The PCR procedures were shown to clearly and specifically detect theilerial types in a large proportion of cattle that do not show smear evidence of disease. Overall, as seen in Figure 16, approximately 90% of smear positive and over 50% of smear negative samples were positive in the p32 and Ikeda assays, confirming the higher sensitivity of the molecular-based tests. From an overall perspective, approximately 15% of smear positive samples with <1% of erythrocytes estimated to contain theilerias were negative on the p32 screening PCR assay (Figure 17) but only 3/96 (3%) samples with ≥1% erythrocytes infected were p32 PCR negative, highlighting the high accuracy of the PCR when still relatively low levels of parasitaemia are detectable on smears. In addition, the procedures adopted indicated that the parallel testing of neat and diluted DNA extract was very effective in detection of PCR positive cattle regardless of their status on blood smears. In fact, testing of the 1:10 extract was more effective alone than testing of neat extract alone, as this yielded 38% more p32 cases, 20% more Ikeda cases, 17% more Chitose cases and 34% more Buffeli cases.

The improved performance of diluted extracts compared to neat extracts suggests PCR inhibitors or excess DNA impairs detection in blood extracts that can be reduced markedly by a simple dilution step. Since PCR positive samples that test smear negative would be expected to contain less theilerial DNA than those that are PCR positive and smear positive, it is of interest that the smear negative PCR positive samples were more readily detected with diluted extracts (Figure 21). In addition, neat extracts were not more effective with smear negative samples, as there was no difference in the proportion of samples detected by neat extracts alone when comparing smear positive and negative samples. Thus neat extracts appeared to perform similarly in instances of high and low DNA loads. Together this data suggests that excess DNA is unlikely to be responsible for the better performance of diluted extracts compared to neat extracts, and it is likely that blood inhibitors are reduced by the dilution step in PCR testing for theileriosis.

6 Conclusions and recommendations

6.1 Distribution of theilerial MPSP types

6.1.1 Testing platform

Conventional PCR testing with separate assays for screening (p32) and for specific MPSP types (Ikeda, Chitose, Buffeli) based on published PCR primer sequences were found to be suitable for these studies, but required minor modifications for improved sensitivity. The modified procedures were shown to be robust, and the screening assay could be implemented as a means of reducing test costs by undertaking specific assays only on screen test positive samples. This was undertaken on most samples from the distribution survey. The value of testing diluted DNA extracts from a conventional kit was clearly evident from results of the significance study, as this process augmented the number of cases detected by PCR, probably by reducing the concentration of inhibitory substances in blood samples. Other steps to save time and decrease variability in the extraction procedure, such as automated systems, would be worthwhile to consider for future testing, if they can shown to be cost-effective.

The amplified products of these PCR assays are relatively large and of similar size, which renders them unsuitable for multiplex conventional assays and unable to be translated directly into real time (quantitative) PCR assays. Future developmental work to validate real time PCR assays that provide a screening and multiplex approach would be worthwhile to reduce testing costs. In addition, this approach offers the possibility of a faster, more sensitive and quantified assay, that could prove also be applied to quantifying infective loads of benign theilerias in blood samples.

6.1.2 State basis

Benign theilerias of the three major MPSP types (Ikeda, Chitose, Buffeli) occur in Queensland, NSW and Victoria with variable prevalence. Among 516 samples collected from 50 cattle herds in these three states, Queensland showed the highest prevalence of benign theilerias by PCR testing (57%) followed by Victoria (34%) and NSW (24%). Chitose was the predominant type in all three states, and was detected in 19-44% of samples. Buffeli was detected in 11-25% of samples from the three states, and Ikeda in 1-12% of samples per state.

In Queensland, Chitose was found in 44% of samples, followed by Buffeli (25%) and Ikeda (11%). In NSW, Chitose was present in 19%, Buffeli in 11% and Ikeda in 12% of samples. In Victoria, Ikeda was rarely detected (1% of samples), whereas Chitose and Buffeli were found in 25% and 22% of samples respectively.

Samples were screened by a PCR that is designed to detect all benign theilerias, based on the p32 MPSP gene where PCR primer target sequences are shared among different MPSP types. Positive results in this assay corresponded with positive results in one or more of the three type specific PCR assays, which suggested that it was unlikely that a significant prevalence of other uncommonly-reported types was occurring in these herds. Based on similar findings in known affected herds, it is unlikely that additional MPSP types are responsible for clinical cases.

6.1.3 Regional basis

MPSP types Ikeda, Chitose and Buffeli were widespread in Queensland and the Ikeda type was evident in 3/5 regions (North, South and South East). The Ikeda type was evident at a

prevalence of 3-20% in these three regions. The other MPSP types were common to four of the five regions, at regional prevalences of 22-70% for Chitose and 17-40% for Buffeli.

In NSW, Buffeli infection was limited to two regions sampled (North Coast and North West), in which Chitose was also at its highest prevalence (44-65%), and Chitose infection was an infrequent finding in the six other regions examined, being only found in single animals in each of three of these regions. Ikeda type organisms in NSW were only detected in the North Coast region, and were found in all 4 herds sampled at a mean prevalence of 77%. Other coastal regions of NSW were not sampled, and may provide similar findings to the North Coast herds.

In Victoria, where sampling was more limited and confined to herds in two regions of Gippsland, only the East Gippsland herds showed evidence of theilerias in 42% of all samples, with Chitose and Buffeli types detected in 31% and 28% of samples. Ikeda infection was only detected in one animal in one herd. This study did not include sampling of herds from West Gippsland, where recent cases of Ikeda infection in clinically affected dairy cattle have been detected in our laboratory.

While it appears benign theilerias are widespread in NSW and Queensland, the important Ikeda type appears more limited in distribution in NSW herds or regions where the prevalence of clinical disease is at low prevalence or not reported. The NSW findings may reflect herds where stock movements from other areas, particularly coastal areas are not present, because the widespread occurrence of clinical theileriosis across NSW regions (including Tablelands, Hume and Lachlan LHPA districts) is at variance with the limited distribution in herds such as those tested here.

In Queensland, while Ikeda infection appears predominant in three regions (North, South and South-East) there is no concurrent evidence of clinical theileriosis. This suggests that either the genetic types in the Queensland herds are less virulent than those encountered in affected NSW herds, or that immunity (or lack of naivity) in Queensland cattle prevents clinical disease, or that environmental or host genotype factors are sufficiently different to preclude disease occurrence despite the presence of virulent strains. Further work is needed to characterise Ikeda strains particularly from herds where disease has or has not occurred.

6.2 Significance of theilerial MPSP types within affected herds introducing livestock

Among the seven affected beef cattle herds studied, infection with the Ikeda type was common to all, whereas Chitose infection was present in six herds and Buffeli in four. The Ikeda type was shown to be the key MPSP type associated with animals of smear positive status in these affected herds. The most valuable clinical and clinical pathology indicators for assessing disease severity of theileriosis in a herd were mucosal membrane colour, PCV and quantification of theilerias in blood smears.

Pale and jaundiced mucosae were associated with clinically affected groups of cattle, and herds showing numbers of cattle with ≥1% erythrocytes containing theilerias were associated with high carrier rates of benign theilerias of Ikeda type, with or without Chitose type. However, from these herds it was evident that for detection of theilerial infection, PCR testing of EDTA blood was more sensitive than traditional smear examinations, which can be subjective and possibly show false positive results particularly where <1% of erythrocytes are considered infected.

In affected herds, Ikeda infection may progress in young calves to high animal prevalence at a faster rate than the other MPSP types, particularly Buffeli type which is generally considered to be avirulent. Direct contact with weaners introduced from coastal areas appeared as a risk

factor, since high prevalence rates were detected in a single time point bleed in purchased adults on one property. The results also indicate that spread within herds is variable, and depends on the time points selected for sampling. Thus, cattle without contact with affected mobs may show no evidence of infection, while in others lack of direct contact with affected cattle had not prevented infection with the Ikeda type.

Within groups showing infection, PCR testing shows repeatedly similar results at multiple samplings at 1 month intervals, so frequent sampling may be unrewarding once high prevalence is established. This is in contrast to smear examinations, which are likely to fail to detect infection on repeat sampling as levels of parasitaemia decline. In clinically normal cattle in affected herds with Ikeda type infection, over half of the smear negative animals can still be detected as being infected with the Ikeda type on PCR testing, while 90% of smear positive cases are expected to give PCR positive results for Ikeda type. This shows there is a large reservoir of infected but clinically normal animals in affected herds.

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9 Appendix

9.1.1 Appendix 1. PCR reaction gradings

Appendix 1.
Examples of PCR reactions to p32 and Ikeda, Chitose and Buffeli in MPSP assays, where reactivity was graded as positive (+), weak positive (w), very weak positive (v) or negative (-). Specific amplicons of 826 bp (Ikeda, Buffeli and 831 bp (Chitose) are expected; arrows indicate 1000 and 500 bp markers.