Comparative Medicine: Investigations into the fields of infectious and zoonotic disease research, and population-level epidemiology

by

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Authors Declaration

I, Claire E. Walls, declare that the work in this thesis is original and was carried out solely by myself or with due acknowledgments. No part of this thesis has been submitted for any other degree.

List of Abbreviations

AHT	Animal Health Trust
AP	Action potential
BBB	Blood-brain barrier
BEF	British Equestrian Federation
BEVA	British Equine Veterinary Association
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$[Ca^{2+}]$	Calcium concentration
CDC	Centres for Disease Control and Prevention, USA
CV	Cardiovascular
DAG	Diacylglycerol
DEFRA	Department for Environment, Food and Rural Affairs
ELISA	Enzyme-linked immunoassay
GDP	Gross domestic product
GEMS	Global Enterics Multi-Centre Study
GEMS-ZED	Global Enterics Multi-Centre Study Zoonotic Enteric Diseases
HBK	HEPES base krebs
HBMECs	Human brain microvascular endothelial cells
HRH	The Home of Rest for Horses
IP_3	Inositol-1,4,5- triphosphate
iRAS	Intracellular renin-angiotensin-aldosterone system
K11777	N-methyl-piperazine-phenylalanyl-homophenylalanyl-vinylsulfone phenyl
KEMRI	Kenyan Medical Research Institute
NCX	Sodium-Calcium exchanger
NED	National Equine Database
PAR	Protease-activated receptor
PAR-AP	Protease-activated receptor activating protein
PCR	Polymerase chain reaction
PIP_2	Phosphatidylinositol-4,5-biphosphate
PLC	Phospholipase C
PTx	Pertussis toxin
RT-PCR	Reverse-transcriptase polymerase chain reaction
SERCA	Sarcoplasmic reticulum calcium ATPase
SR	Sarcoplasmic reticulum
WHO	World Health Organisation
ZED	Zoonotic Enteric Diseases Study

General Introduction

This thesis is formed by the amalgamation of three scientific papers (unpublished) covering differing subjects within the fields of comparative and veterinary medicine. The specific aims of each project, listed below, were diverse. Each was designed to involve a brief literature review with hypothesis formulation, an investigative phase, and finally the production of a document for inclusion in this thesis. Projects 1 and 3 were practical, 'wet-lab' investigations into aspects of human and animal disease in the developing world, whereas project 2 involved analysis of existing data. All three projects contributed to the bank of knowledge on human and animal disease, and contained elements concerning infectious disease research. Over the past decades a greater awareness for the need to combine research from medical and veterinary spheres has arisen, due to the geographical and genetic impermanence, and the connectedness of these populations. With this principle in mind, the projects included in this thesis feature elements of epidemiology, laboratory-based investigative methods and data analysis which are relevant to the study of disease in both the human and animal medical fields.

The specific research questions posed were as follows:

Project 1

- Can a known cathepsin L-like inhibitor (K11777) affect the generation of spontaneous Ca^{2+} 'waves' in isolated adult rat cardiomyocytes exposed to the supernatant from suspensions of *Trypanosoma brucei brucei*, suggesting that this parasite produces a cathepsin L-like protease which affects cardiovascular tissues?
- Does the use of the protease-activated receptor (PAR) activating-peptide SLIGRL-NH_2 alter the production of spontaneous Ca^{2+} 'waves' in isolated adult rat cardiomyocytes, suggesting a role for PAR 2 in 'wave' production?

Project 2

- Did the profile of reported disease in the equine population of northern UK alter between the 1960s and the 1990s? Can hypotheses be developed about why any changes may have occurred?

Project 3

- What is the prevalence of *Giardia* and *Cryptosporidium* in a small subset of the domestic animal faecal samples submitted to the Kenyan Medical Research Institute (KEMRI) as part of the 'Global Enterics Multi-Centre Study Zoonotic Enteric Diseases' (GEMS-ZED) project?
- Using a small subset of the existing data in the GEMS-ZED project, what is the prevalence of the major zoonotic enteric pathogens in diarrhoeic and non-diarrhoeic children and the domestic animals in their vicinity, in an area of rural western Kenya?
- Can these figures be used to generate strategies to reduce the morbidity and mortality of young children from diarrhoea in future?

CHAPTER 1 - Trypanosomiasis and the Heart: Evaluation of the effects African Trypanosomiasis on isolated adult rat cardiomyocytes

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CHAPTER 1 - Trypanosomiasis and the Heart: Evaluation of the effects African Trypanosomiasis on isolated adult rat cardiomyocytes

Abstract

Human African Trypanosomiasis causes cardiac rhythm disturbances through hitherto unknown mechanisms. It is known that the presence of such protozoan parasites or simply their supernatant has excitatory effects on isolated cardiomyocytes, seen as increased Ca^{2+} wave occurrence. We investigated the theory that the protozoa are able to release a cathepsin L-like product into solution which is the initiator of such waves. Adult rat left ventricular cardiomyocytes were isolated in solution before [Ca^{2+}] was raised to physiological extracellular concentration. The cells were then exposed to pH controlled samples of supernatant, with or without a known cathepsin L-like protease inhibitor (K11777), and all were subjected to population studies of wave incidence over 60 second timeframes.

Results

Baseline percentage of spontaneously waving cells in mock extracellular solution was found to be significantly lower than with the presence of Trypanosome supernatant. Addition of K11777 led to a significant reduction in spontaneity toward baseline. These results suggest that *T. b. brucei* produces a cathepsin L-like cysteine protease which causes increased incidence of Ca^{2+} waves.

1.1 Introduction

Protozoan parasites of the genus *Trypanosoma* are responsible for a great deal of morbidity and mortality in both humans and veterinary species in many parts of the world. In particular, sub-Saharan Africa suffers heavily from the economic and welfare impacts of the causative agents of Human African Trypanosomiasis (HAT), *T. brucei rhodesiense* and *T. brucei gambiense* (East, and West African Trypanosomiasis, respectively). The vast majority of HAT cases are of the West African form (Blum *et al.* 2006). HAT is spread by bites from

infected Tsetse flies (*Glossina* spp.) and occurs in two clinical stages. In the first, or 'haemo-lymphatic' stage, patients typically suffer lymphadenopathy, intermittent fever, pruritis and arthralgia (Blum *et al*. 2006). Upon penetration of the parasite beyond the blood-brain barrier (BBB), the second 'meningo-encephalitic' stage commences. Symptoms include night insomnia, daytime somnolence (from which the disease acquires the name 'Sleeping Sickness'), headaches, disturbed behaviour, epiletiform attacks and other markers of neurological and psychological derangement (Blum *et al.* 2006). Without treatment most victims succumb to the disease over a period of months to years. It is thought that at present, 17,500 new cases of HAT are diagnosed yearly, with a prevalence of 50,000 to 70,000 in the 36 countries at risk (www.who.int 2010). Difficulties in obtaining epidemiological data from less developed but geographically expansive parts of the continent mean that the current incidence and prevalence data are likely to be underestimates. Current treatments are inadequate due to high levels of toxicity, their failure to rid the patient of all parasites despite prolonged treatment regimens, and the emergence of parasite resistance.

Cardiac arrhythmias are reported in up to 71% of cases of HAT, but are thought not to contribute significantly to mortality (Blum *et al.* 2008). Electrocardiographic alterations observed include prolonged QTc intervals, repolarisation changes and low voltages, all of which are consistent with peri-myocarditis and can be seen early in disease progression. Studies have shown that up to 72% of patients have histological changes to the heart found at post-mortem (Blum *et al.* 2008).

It has long been known that alterations in Ca^{2+} handling in heart cells can lead to spontaneous contractile events known as 'Ca^{2+} waves' in individual cells, which are implicated in various arrhythmias (Cheng and Lederer 2008). Recently, it was discovered that the presence of *Trypanosoma* species could elicit a high frequency of such waves when introduced to suspensions of isolated adult dog cardiomyocytes (Barr *et al.* 1996). Interestingly, the same effect is noted on addition of only the supernatant from cultured parasites, without the presence of protozoa (Barr *et al.* 1996). It was hypothesized that the high degree of spontaneous activity seen was due to the binding of some unknown excretory/secretory product produced by the Trypanosomes, to cardiomyocyte receptors, leading to an intracellular signalling cascade resulting in a wave. This product was thought to be a cathepsin L-like cysteine protease (Barr et al. 1996; Grab et al. 2009).

1.1.1 Physiological Excitation-Contraction Coupling

Intracellular Ca^{2+} control is central to the regulation of contraction and relaxation of individual cardiomyocytes, and therefore also the synchronization of heart chamber contraction. At rest, the cytoplasmic Ca^{2+} concentration is maintained at much lower concentrations (100 nM) compared with the extracellular domain (Bers and Guo 2005). This is achieved by the sequestration of Ca^{2+} within the sarcoplasmic reticulum (SR)(3 mM l^{-1}), an active process performed by the SR Ca^{2+} ATPase pump (SERCA), and to a much lesser degree through slow Ca^{2+} uptake into mitochondria, or through extrusion by sarcolemmal Ca^{2+} ATPases (Bers and Guo 2005; Bers 2008; Cheng and Lederer 2008; Eisner 2010). Na^{+}-Ca^{2+} exchanger (NCX) pumps on the cell surface use energy generated from the flow of Na^{+} down its concentration gradient to move Ca^{2+} in the opposite direction. This flow is reversible, depending upon concentrations of both Ca^{2+} and Na^{+}, and the voltage experienced at the cell membrane (Bers and Guo 2005).

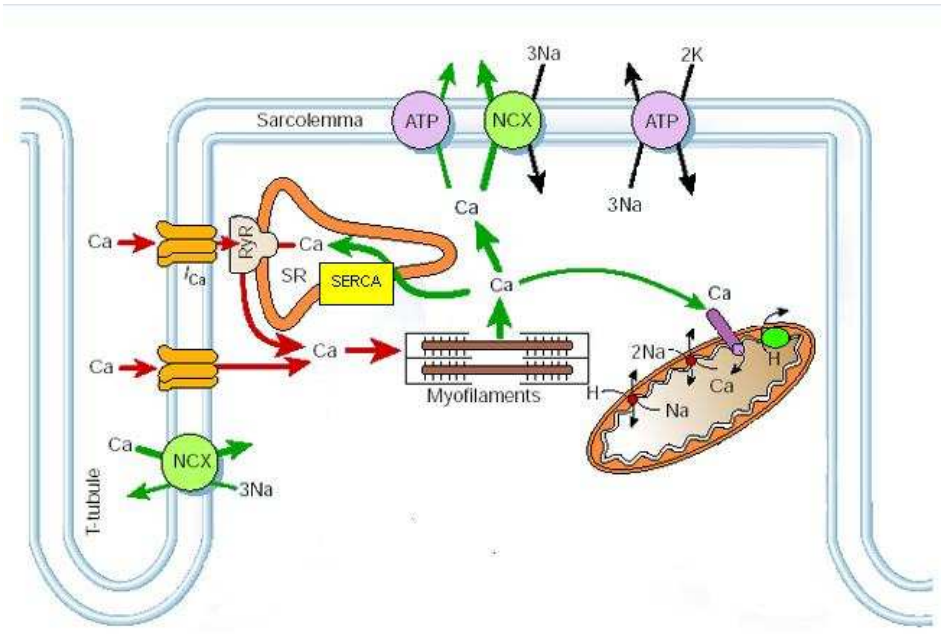

Figure 1.1 Diagrammatic representation of the events occurring during an action potential. Diagram adapted from Donald M. Bers (Bers 2002).

Upon arrival of an action potential (AP) at the sarcolemma, L-type voltage-gated Ca^{2+} channels, located at junctional clefts within functional units known as 'couplons', allow a

surge of Ca^{2+} to enter the cell, raising the intracellular Ca^{2+} concentration to around 10 M (Bers and Guo 2005). Within a couplon, the 25 or so L-type Ca^{2+} channels are clustered close to up to 200 Ryanodine receptors (RyR). These RyRs allow efflux of Ca^{2+} after being induced by the presence of Ca^{2+} itself: a process known as Ca^{2+}-induced Ca^{2+} release (CICR). Due to the proximity of L-type Ca^{2+} channels and RyRs the initial Ca^{2+} influx triggers a much greater flux of Ca^{2+} into the cytoplasm from the SR store. The temporarily high voltage produced also reverses the action of the NCX briefly to favour Ca^{2+} influx, but an increasing intracellular concentration quickly halts this (Bers and Guo 2005). A high concentration of Ca^{2+} is then free to initiate cross-bridge cycling by binding to myofibrils. Cessation of Ca^{2+} influx from L-type receptors occurs due to the negative feedback afforded to the receptor from the presence of Ca^{2+} ions at the intracellular domain (Bers and Guo 2005). Thus the L-type current itself can inhibit further Ca^{2+} influx, and also the SR Ca^{2+} release will play a part in halting entry of Ca^{2+} from outside the cell. The mechanism of termination of Ca^{2+} release from the SR is not completely understood, but is thought to be dependent upon the $[Ca^{2+}]$ within the SR (Bers 2002). The intracellular $[Ca^{2+}]$ needs then to be returned to a resting level in order that the cell can relax and be ready for subsequent contractions. This is done by the SERCA and NCX, and to a much lesser degree by mitochondrial Ca^{2+} uniporter uptake, and extrusion via the sarcolemmal Ca^{2+} ATPase pump (Bers 2002).

1.1.2 Ca^{2+} Sparks and Waves

In diastole, overloading of the SR with Ca^{2+} such that the concentration within exceeds 100 µM of unbound Ca^{2+} is known to lead to 'leakage' of Ca^{2+} into the cytoplasm (Cheng and Lederer 2008). This Ca^{2+} can then trigger CICR from local RyRs, causing a surge of ions which, if large enough, can cause further activation at neighbouring couplons sited about 0.7 µm away (Barr et al. 1996; Cheng and Lederer 2008). This cascade of non-voltage-dependent Ca^{2+} release causes cross-bridge cycling and thus cell shortening in the same manner as APs during systole, and lasts around 10 ms. Globally within the cell the movement produced is distinct from the effect of an AP, in that contraction occurs at a single point before spreading out to cover the cell. During an AP, the entire cell contracts uniformly. The events described occurring at a single couplon are termed a 'spark', but if a spark is able to propagate itself along the length of a cell it is known as a 'wave' (Keizer and Smith 1998; MacQuaide et al. 2009). Ca^{2+} sparks occur in healthy cardiomyocytes at a low frequency, but rarely succeed in

causing ‘full blown’ waves. A wave has consequences for the heart as a whole if it can lead to propagation of similar waves in neighbouring cells, thus forming a syncytium of dysregulated contraction which will disrupt the choreography of normal systolic events. Waves have been implicated in many arrhythmias such as delayed after-depolarisations (DADs) and early after-depolarisations (EADs) (Clusin 2003; Cheng and Lederer 2008). EADs are depolarisations which commence during the plateau of an AP, hence are more likely to occur if there is prolongation of AP duration. They are mediated through the L-type cell surface Ca^{2+} channels, and are clinically relevant due to their strong association with torsades des pointes ventricular tachycardia (Clusin 2003). In conditions of SR Ca^{2+} overload, diastolic release of Ca^{2+} from the SR causes a reversal of directionality of the NCX ion transport such that an inward current is temporarily favoured. This inward current is termed a DAD, and if sufficient in amplitude, can lead to propagation. Ca^{2+} is able to pass through gap junctions to propagate CICR and thus arrhythmia in the heart as a whole (Barr *et al.* 1996).

The likelihood of Ca^{2+} waves occurring in cardiomyocytes can be increased by various mechanisms. As mentioned previously, an abnormally high [Ca^{2+}] within the SR allows RyRs to become ‘leaky’ which can set up the Ca^{2+} release necessary for a wave. But what causes SR overloading? A high extracellular [Ca^{2+}] leads to inward movement of Ca^{2+} through the NCX, which in turn causes more Ca^{2+} to be taken up by SERCA into the SR. These events occur in diseases such as heart failure, other ischaemic states and digitalis toxicity (Cheng and Lederer 2008; Xie and Weiss 2009). Genetically inherited defects in regions coding for RyRs are known causes of excess leakage of Ca^{2+} from the SR, and thus arrhythmias in humans (Xie and Weiss 2009).

1.1.3 Proteases and PARs

Proteases are ubiquitous hormone-like signalling molecules with wide ranging effects in the mammalian body. Trypanosome species can produce their own proteases which can allow them access to the central nervous system, and which play roles in disease pathogenesis and parasite growth (Nikolskaia *et al.* 2006). Examples are the cathepsin L-like cysteine protease ‘brucipain’ produced by *T. brucei gambiense*, ‘rhodesain’ from *T. b. rhodesiense* and ‘cruzain’ produced by *T. b. cruzi*. Indeed it is now thought that brucipain is essential to *T. b. rhodesiense*, shown by the failure of RNAi of this protease to produce viable parasites (J.

Mottram, pers. Comm.)(Nikolskaia *et al.* 2006). Proteases can influence intracellular [Ca^{2+}] through the activation of protease-activated receptors (PARs).

PARs are a family of G-protein coupled, 7-transmembrane helix cell surface receptors, four of which have been discovered to date (Hansen *et al.* 2008). Genes encoding PARs 1, 2 and 3 are localized on chromosome 5q13, with PAR 4 being structurally similar but located on chromosome 1qp12 (Barnes *et al.* 2004). Activation of these receptors can be achieved in two ways. Firstly, proteolytic cleavage of a short amino acid sequence at the extracellular surface of the molecule frees a tethered ligand structure. This ligand is then able to contact an activation site, leading to a conformational change in the PAR, and a downstream activation cascade. This is an irreversible mechanism, terminating in receptor internalisation (Bohm *et al.* 1996). No function for the cleaved portion of the PAR has yet been discovered (Barry *et al.* 2006). Alternatively, short amino acid sequences called PAR-activating peptides (PAR-APs) are able to mimic the tethered ligand and can reversibly bind directly to the extracellular activating domain (Bohm et al. 1996; Hansen et al. 2008). Studies of the effects of *T. cruzi* on canine cardiomyocytes proved that the receptors involved in the wave response were G-protein linked, by noting the effect of pertussis toxin (PTx) on wave frequency. PTx causes ADP-ribosylation of some of the G-protein α-subunits which blocks signal transduction. The addition of PTx was able to completely abolished Ca^{2+} waves in these studies (Barr *et al.* 1996). Following activation, PARs are internalised into endosomes and subsequently degraded within lysosomes (Bohm et al. 1996; Trejo 2003; Barry et al. 2006). Cleavage and internalisation of PARs is one mechanism of desensitisation to protease-induced activation, but others including receptor phosphorylation leading to uncoupling of G-proteins, and inhibition of downstream signalling events also occur (Bohm *et al.* 1996). It has been shown that desensitisation occurs within 2 min of stimulation, but also that cells resensitise over a period of 30-60 min (Bohm *et al.* 1996). This resensitisation is due to mobilisation of stores of PAR 2 from post-Golgi organelles initially, followed by stores within the Golgi itself. If further stimulation occurs, the cell must manufacture nascent PAR 2 to replenish cell surface stores and remain sensitive (Bohm *et al.* 1996).

Each of the PARs discovered to date can also be irreversibly antagonised with known molecules, most of which block activation by cleavage of the tethered ligand from the receptor, thus rendering the PAR unable to ‘self-activate’. However, PAR-APs can overcome

this inactivation by binding directly to the extracellular binding site. Alternatively, cleavage of one of the exposed portions of a transmembrane loop leads to receptor inhibition (Barry *et al.* 2006).

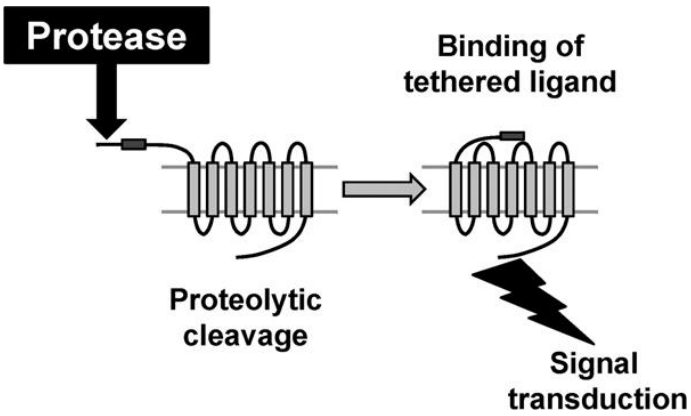

Figure 1.2 Diagrammatic representation of PAR activation by a protease. Signal transduction begins with G-protein activation. Adapted from a diagram from Critical Care Forum Online (www.ccforum.com).

PARs are distributed throughout the body and can be responsible for very varied, occasionally antagonistic actions. For instance, activation of PAR 1 can lead to analgesic effects, whereas PAR 2 has been implicated in producing pro-algesic effects. PAR functions include the modulation of nociception, platelet activation, maintenance of vascular tone, bronchospasm/relaxation and inflammation (Sambrano et al. 2000; Barnes et al. 2004; Asfaha et al. 2007; Dale and Vergnolle 2008; Hansen et al. 2008; Pinet et al. 2008). PAR 2 is thought to be the receptor which is involved in the Trypanosome wave response (Grab *et al.* 2009). Activation of PAR 2 leads to the activation of Phospholipase C (PLC), with the subsequent production of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-biphosphate (PIP_2). IP_3 would then be free to bind to its receptors on the SR, initiating release of Ca^{2+} from intracellular stores. Indeed, it has been shown that IP_3 and PLC are found at higher concentrations in cells exposed to Trypanosome supernatant (Barr et al. 1996; Grab et al. 2009). This increase in intracellular [Ca^{2+}] could predispose the SR to overloading, and thus increase the likelihood of Ca^{2+} wave formation.

Table 1.1 Endogenous activators, activating peptides and inhibitor molecules active against each protease-activated receptor (Trejo 2003; Barry et al. 2006; Ramachandran and Hollenberg 2008; Strande et al. 2008).

PAR Type	Activators	PAR-APs	Inhibitors
PAR 1	Thrombin	SFLLRN	SCH-203099
	Cathepsin-G	SFFLRN	SCH-205831
			SCH-5303048
			ER-112787
			BMS-200261
			RWJ-56110
			RWJ-58259
PAR 2	Trypsin	SLIGRL	ENMD-1068
	Tryptase	SFLLRN	
	Factor Xa	SLIGKV	
		2-furoyl-LIGRLO	
PAR 3	Thrombin		
PAR 4	Thrombin	GYPGQV	Trans-cinnamoyl-YPGKF
	Trypsin	GYPGKF	P4pal-10
	Tryptase	AYPGKF	
	Cathepsin-G		

1.1.4 iRAS Theory

Proteases might alternatively lead to Ca^{2+} waves through activation of the intracellular Renin-Angiotensin-Aldosterone (iRAS) system. All of the components of this system can be found in cardiomyocytes, albeit at low levels physiologically (Kumar *et al.* 2008). Cathepsins can activate the production of intracellular Angiotensin II which leads to an increase in Na^{+} extrusion, which in turn could increase the influx of Ca^{2+} ions via the NCX (Gavras and Gavras 2002; Iravanian and Dudley, Jr. 2008). Increase in Angiotensin II has also been shown to increase AP duration and repolarisation dispersion, which are known to be arrhythmogenic (Perrier et al. 2005; Iravanian and Dudley, Jr. 2008; Kurokawa and Abriel 2009). The pro-arrhythmic effects of Aldosterone include increase in the Ca^{2+} influx size, longer AP duration and an increase in the expression of L-type Ca^{2+} channels (Perrier et al. 2005; Kurokawa and Abriel 2009). Overall, the effect of activating iRAS in the heart is to produce cardiac hypertrophy and fibrosis (Gavras and Gavras 2002). Investigation of this hypothesis was beyond the scope of this study.

It is hypothesised that the Trypanosome product causing the Ca^{2+} wave response in adult rat cardiomyocytes is a cathepsin L-like protease, which interacts with PAR 2 receptors on cardiomyocytes leading to wave formation (Nikolskaia et al. 2006; Grab et al. 2009). In this

study, the ability to block this action with a known irreversible inhibitor was used to study the effects on spontaneous wave production. K11777 is an irreversible cathepsin L-like cysteine protease inhibitor, known to inhibit the crossing of human brain microvascular endothelial cells (HBMECs), (a human BBB model system) by *T. b. gambiense* (Nikolskaia *et al.* 2006).

This study had two overall aims; a) to investigate the effects of the cathepsin L-like protease inhibitor K11777, and thus determine whether a cathepsin L-like protease product produced by *Trypanosoma* was the cause of increased Ca^{2+} waves, and b) to utilize the PAR-AP SLIGRL-NH_2 to determine whether PAR 2 may be involved in spontaneous wave production of isolated adult rat cardiomyocytes.

1.2 Materials and Methods

1.2.1 Cell Isolation

Adult male Wistar rats (225-300 g) were euthanized via a Schedule One procedure in accordance with the UK Animals (Scientific Procedures) Act 1986. Hearts were quickly excised and placed into ice cold Krebs and gently squeezed to expel the majority of blood. Any extraneous tissues were rapidly dissected away, the aorta isolated, and the hearts weighed. Hearts were rapidly cannulated onto a Langendorff retrograde perfusion system via the aorta, and secured in place. All solutions perfusing the heart were maintained at 37.0-37.5^{0}C using a water bath, and the rate of perfusion was maintained at 6 ml/min. The hearts were perfused with Krebs solution (see composition below) for 5 min to flush out all residual blood. After this time, the hearts were perfused for 10 min with Krebs solution supplemented with 0.83 mg ml^{-1} Collagenase and 8.3 µg ml^{-1} Protease. Finally BSA solution at 6.5 mg ml^{-1} was perfused for 3 min 20 s. Following this perfusion, the hearts were submerged in warm BSA, the atria and right ventricle dissected away, and the remaining tissue dissected into small, roughly uniform pieces. The tissue pieces were gently agitated in BSA for on average 15 mins to yield a single cell suspension. The [Ca^{2+}] of the cell suspension was increased in stepwise increments to a final concentration of 1 mM for Trypanosome experiments, or 1.4 mM for PAR-AP experiments. The cells were allowed to settle into a pellet by gravity before being resuspended in HBK solution.

1.2.2 Solutions

All solutions were prepared freshly on the day of an experiment, with pH corrected at room temperature. All concentrations are in mmol/L unless stated otherwise.

Base Krebs - 120 NaCl, 20 HEPES, 5.4 KCl, 0.52 NaH_2PO_4, 3.5 $MgCl_26H_2O$, 20 Taurine, 10 Creatine, 11.1 glucose anhydrous, adjusted to pH 7.68.

Enzyme solution – Base Krebs supplemented with 0.83 mg ml^{-1} Collagenase (Type 1, Sigma Chemical) of activity 316 u mg^{-1}, and 8.33 µg ml^{-1} Protease (Type XIV, Sigma Chemical) at 4.8 u mg^{-1} activity.

BSA solution – Base Krebs supplemented with 6.5 mg ml^{-1} BSA (Sigma Chemical).

Calcium - The Ca^{2+} concentration was varied in the isolated cell suspensions by adding known volumes of 1 M $CaCl_2$ solution (BDM).

Mock extracellular solution (termed HEPES Base Krebs (HBK)) - was used to resuspend isolated heart cells before commencing population studies, its composition was as follows (mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 5 HEPES, 11.1 glucose anhydrous, 1 $CaCl_2$, adjusted to pH 7.4.

K11777 was a kind gift from Professor Jeremy Mottram.

PAR-AP - The PAR-AP SLIGRL-NH_2 (Tocris biosciences) was introduced to cell suspensions by adding known volumes of 10 M solution.

1.2.3 Trypanosoma species

Populations of *Trypanosoma brucei brucei* strain Lister 427 were maintained axenically in logarithmic growth phase in a modified version of HMI9 medium (Hirumi and Hirumi 1994), the medium that is widely used to culture bloodstream (mammalian form) trypanosomes. The modified recipe is as follows (P. Voorheis, pers. comm.): IMDM (Iscove's Modified

Dulbecco's Medium, Invitrogen) supplemented with; 1 mM hypoxanthine, 50 µM bathocuproinedisulfonic acid, 1.5 mM cysteine, 2 mM sodium pyruvate, 160 µM thymidine, 200 µM 2-mercaptoethanol, 1.4 mM glucose, 125 µM adenosine, 125 µM guanosine, 30 µg/ml kanamycin, 1 mg/ml methyl cellulose. Parasites were cultured in HMI9 with 20% v/v Serum Plus, a serum supplement containing Foetal Bovine Serum (SAFC Biosciences) in a humid incubator at 37°C, 5% CO_2. Counting of parasites was done in triplicate using an improved Neubauer Haemocytometer. Supernatant was prepared by spinning cultured trypanosomes at 857 g for 10 min, and the supernatant was subsequently removed by pipette. To ensure there was no contamination with live trypanosomes, supernatant was further filtered using a 0.2 µm syringe filter (Sartorius Stedim). Control medium was treated in an identical manner to the supernatant (spun and filtered). Inhibitor K11777 was added after spinning and filtration, at a concentration of 10 µM. 0.1% DMSO was added to no-inhibitor controls. Live parasites were re-suspended in approximately 10 ml of medium and recounted in triplicate using the improved Neubauer Haemocytometer. Suspensions of parasites and removed supernatant were adjusted to the pH of the control HMI9 batch used for each experiment.

1.2.4 Data Recording - Population Studies

Baseline spontaneity of cardiomyocytes was assessed by microscopic 'population studies' of the incidence of waves produced over 60 s by rod-shaped ('live') cardiomyocytes over ten microscope fields at the 10X objective. Samples of the cardiomyocytes in HBK were incubated for 30 min at room temperature with supernatant or growth media, with or without K11777 added. Population studies were performed to compare the spontaneous wave activity of cardiomyocytes when exposed to *Trypanosoma* supernatant, or growth media. The effect of adding the inhibitor K11777 was assessed, and appropriate control solutions were also used. All population studies were controlled for suspension pH and time. Control solutions for K11777 were media plus the equivalent volume of vehicle (DMSO). Chi-square tests were used where appropriate to assess differences in wave production.

1.2.5 PAR-AP Experiments

PAR-AP experiments were conducted after raising the $[Ca^{2+}]$ of cell suspensions to 1.4 mM and assessing baseline spontaneity initially. Known volumes of 10 M SLIGRL-NH_2 were

incubated with cardiomyocytes for either 5 min or 30 min, and effects on spontaneous wave production recorded.

1.3 Results

The weight of the adult rat hearts used was 1.54 g ± 0.422 g. Following isolation of a suitable population of live ('rod form') adult rat cardiomyocytes, the proportion of cells exhibiting at least one Ca^{2+} wave over 60 seconds was recorded as a proportion of total live cells, for ten fields at the 10X objective ('baseline spontaneity'). Cell suspensions containing growth media with either DMSO or K11777, in the absence of supernatant, produced a level of wave production which was not significantly different from the baseline measure. Wave production in cell suspensions with supernatant, but not K11777, was significantly increased above all other suspensions. Upon addition of K11777 to those cell suspensions containing supernatant, wave production occurred in a similar proportion of cardiomyocytes to baseline and media suspensions.

1.3.1 Inhibitor K11777

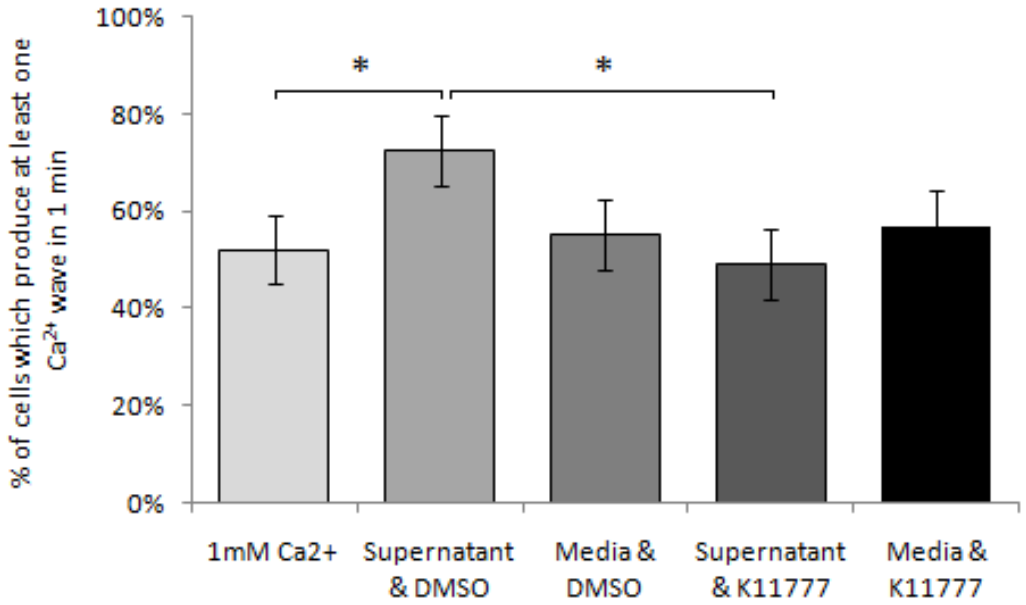

Figure 1.3 Comparison of wave incidence over 60 s with combinations of growth media, supernatant and cathepsin L-like cysteine protease inhibitor K11777. Cardiomyocyte suspensions were incubated with each solution for 30 min prior to wave assessment. (***** $p < 0.05$). Supernatant produced a predictable rise in wave production. Introduction of K11777 reversed this trend back toward baseline, n=5.

1.3.2 PAR-AP Experiments

As Figure 1.4 shows, no significant alterations in wave production were seen following addition of the PAR 2 activating peptide SLIGRL-NH_2 at 10 µM, 100 µM, 300 µM, or 600 µM at any time. Extended incubation times of 30 min failed to produce any alteration in wave production. Incidence of waves in cell suspensions devoid of any Trypanosome product or medium remained the same over the period of the experiments (approx. 5 hrs), thus ageing change or time-dependant degradation of cardiomyocytes was not responsible for any of the results produced.

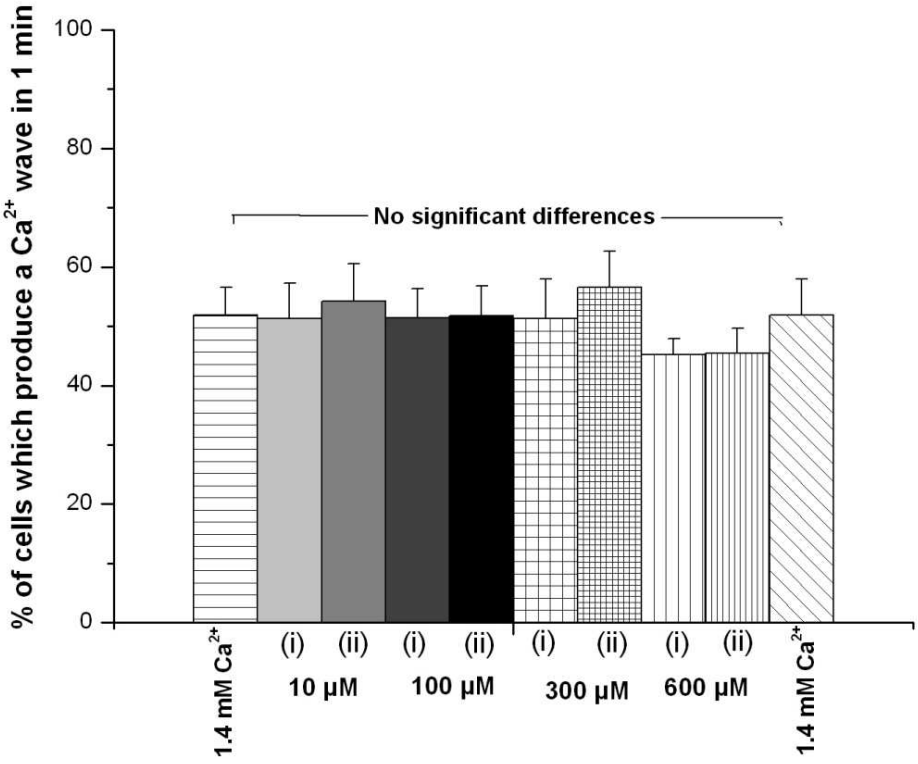

Figure 1.4 Comparison of wave production after incubation of isolated cardiomyocytes with varying concentrations of SLIGRL-NH_2 for (i) 5 min or (ii) 30 min. No concentration of SLIGRL-NH_2 produced an increase in wave incidence at any time. No appreciable degradation in cardiomyocytes was observed over time, as shown by the similarity in wave incidence at the beginning and end of the experiment.

1.4 Discussion

Barr et. al discovered that the presence of *Trypanosoma cruzi* supernatant caused a significant increase in the occurrence of diastolic Ca^{2+} waves in isolated adult cardiomyocytes (Barr *et al.* 1996). This phenomenon was also observed using other Trypanosome species. It was thought that the cause of this effect was excretion/secretion of a protease product from the parasite into the surrounding medium. Trypanosomes of various species are known to employ protease excretions in pathogenic mechanisms for invasion and adhesion. Indeed, it seems likely that invasion of the CNS and thus the major morbidity caused by *T. brucei* species is due to production of a cysteine protease which disrupts the normal Ca^{2+} handling of the cells of the BBB (Nikolskaia *et al.* 2006). In this study we demonstrate that the protease produced by *T. b. brucei*, a closely related organism to the causative agents of HAT, is a cathepsin L-like

cysteine protease, whose effects on isolated cardiomyocytes can be abrogated by addition of the inhibitor K11777.

Sabri et. al. showed that IP_3 production is altered by the addition of the PAR-AP SLIGRL-NH_2 after 5 min at a concentration of 300 µM (Sabri *et al.* 2000). This study set about to assess the effects of this peptide on wave production in a time and concentration dependant manner. Although a small increase in wave production was observed above baseline in those experiments using 300 µM peptide, this was not significant. The inherent sensitivity of the aforementioned population studies to detect PAR activation is low. Also it is unknown what proportion of Ca^{2+} handling in adult cells is due to IP_3 production rather than RyRs, compared with neonatal cardiomyocytes. These factors taken together imply that the failure to see any alteration in wave production using PAR-APs, does not necessarily mean that PAR 2 is not involved. Rather, it suggests that more sensitive techniques such as single cell perfusion techniques and IP_3 assays may be of benefit in future. Repetition of these experiments and use of the reverse peptide LRGILS-NH_2 was beyond the scope of this study.

HAT is an infectious disease of major importance, whose main pathogenesis lies in the neurological damage caused by entry into the CNS of the causative organism. A significant contributor to this disease picture is the cardiovascular derangement seen in the majority of HAT cases (Blum *et al.* 2006). The arrhythmogenic potential of Trypanosomiasis comes further into focus when novel drug therapies are themselves a cause of cardiac rhythm disturbance. Although not usually fatal alone, if Trypanosomiasis arrhythmias are worsened by drug therapy, death through heart failure becomes a possibility. Current drug treatments for both first and second stage HAT carry a high degree of toxicity, are not completely effective, have complicated and lengthy administration regimens, and have conferred some degree of drug resistance within parasite populations (www.who.int 2010). Active investigation into possible new therapies is being undertaken in the hope that they will yield higher efficacy with lower toxicity among patients.

Protease inhibitors are currently used as treatments for diseases such as HIV, parasitic infections such as Giardiasis, and neoplasia. One of the difficulties in producing pharmacologically effective protease inhibitors lies in targeting only the pathogenic uses of proteases, and sparing those physiological functions which may be many and diverse.

Cathepsin L-like molecules are ubiquitous in the mammalian body, making drug targeting a difficult but crucial stage in development.

Ca^{2+} handling appears to be central to the mechanism of CNS invasion employed by those species of Trypanosomes whose life-cycle includes such a stage (Nikolskaia *et al.* 2006). It is not known exactly how this alteration in Ca^{2+} handling allows penetration beyond the BBB, but it is clear that cysteine proteases are involved (Nikolskaia *et al.* 2006). It remains to be seen whether the same papain-like cysteine protease which causes BBB traversal, also causes cardiac arrhythmias. The implications would be that a common novel drug therapy may be able to prevent both the neurological, and the cardiac manifestations of HAT, by inhibiting the action of the particular cysteine protease involved.

CHAPTER 2 - The Changing Profile of Equine Disease in Great Britain from the 1960s to the 1990s

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CHAPTER 2 - The Changing Profile of Equine Disease in Great Britain from the 1960s to the 1990s

Abstract

Two survey-based studies of equine disease in the UK gathered thirty years apart were compared on the basis of disease category prevalence. The first study, published in 1965, was conducted by the British Equine Veterinary Association and covered the whole of mainland UK and Ireland. The second dataset was obtained from The Home of Rest for Horses Study, which surveyed Scotland and northern England in the mid-1990s. Musculoskeletal and alimentary diseases were the most important categories of disease in both studies, but there were significant differences seen in both the composition of the horse population in terms of breed type, and the profile of disease between the studies. The predominant horse type in the BEVA study was the pony, whereas the HRH study reported a more even distribution of ponies, hunter-type horses and racehorses within the population. Disease categories which were more commonly diagnosed in the HRH versus the BEVA study included musculoskeletal and systemic disease. Those categories which had were less commonly diagnosed comprised alimentary and respiratory disease.

2.1 Introduction

Professional and recreational equestrianism are popular pursuits within the UK. Studies estimate that the current number of horses in Great Britain lies between 600,000 and 1 million (Mellor et al. 1999; E.J.Leckie 2001; BHIC Ltd. 2009). It is estimated that up to 270,000 people are directly or indirectly employed in equestrianism and associated support industries, which have an annual turnover (all sources of revenue combined) in the region of £3.4 billion (BHIC Ltd. 2009). The Animal Welfare Act 2006 dictates that owners and carers of equines in the UK have a legal responsibility to safeguard their welfare. Knowledge of population disease status and how it has changed over time in association with trends in management and treatment would be useful and informative to help improve future equine welfare. Reliable population data is necessary to allow robust epidemiological studies to inform on the control

and prevention of disease. Currently there is no such information available for the UK herd as a whole.

In 1997 Mellor *et al.* published results from a three year study which set out to define the size and demographics of the horse population of Scotland and northern England, along with the prevalence of diseases occurring over a one-year period as seen by first opinion veterinarians (HRH study). A study conducted by the British Equine Veterinary Association (BEVA) in the early 1960s recorded similar information on disease occurrence in horses in England, Scotland and Ireland, also over a one year period (BEVA study). These studies stand alone as the only large-scale investigations into disease prevalence within a proportion of horses in the UK. The aim of this paper was to compare these datasets to discover what, if any, changes had occurred in disease prevalence over the thirty years between the BEVA and HRH studies.

2.2 Materials and Methods

2.2.1 BEVA Study

During the British Equine Veterinary Association Survey of Equine Disease, BEVA members from 70 practices and clinics across Scotland, England and Ireland were asked to provide monthly feedback detailing every case of equine disease attended over the period from June 1962 to May 1963, and to state the number of horses under the care of their practice. Returns were grouped into four geographical areas: the 'North' including Scotland and England as far south as Hull and Liverpool, the 'Midlands' extending from the North region to Bristol and Colchester, the 'South', and Ireland. The published data comprised lists of diagnoses, grouped into thirteen disease categories, for which the number of cases reported in racehorses, hunters, ponies and heavy horses were included. The authors did not draw any conclusions from the data in the publication. The methods of data collection and categorisation into disease groupings or breed type were not stated in the paper, and did not exist in any linked resources, such that the robustness and reliability of the figures stated could not be assessed.

2.2.2 HRH Study

The Home of Rest for Horses, founded in 1886, is a charitable organisation concerned with equine welfare issues. In the 1960s the organisation began making grants to veterinary practices and universities to fund capital building projects and research designed to benefit equine health and welfare. Unsurprisingly, much of the research on equine health and disease was conducted in University teaching hospitals, whose case load is not necessarily representative of that of the general equine population. In the 1990s it was agreed that obtaining detailed survey-based information on the health and husbandry of the general population of horses would benefit all equidae, by revealing areas of horse care which required further research. To this end, The Home of Rest for Horses funded a project to collect and analyse data on the total size, composition and geographical distribution of the equine population of Scotland and northern England. In addition, data were gathered on the management, activity level and disease prevalence of horses in the area. The study began in October 1992 and used a sentinel practice-based approach to data gathering. Four surveys of specifically recruited first opinion veterinary practices were conducted. The detailed design of the survey programme is given in Mellor *et al*. (1999). For the collection of disease diagnosis data, 25 randomly chosen veterinary practices, from a census of all veterinary practices in Scotland and Northern England involved in equine work, were invited to be sentinel practices. Individual clinicians within each practice agreed to complete and return ten specially designed 'case sheets' on a quarterly basis for one year, for the first ten equine animals attended for whatever reason each time after receiving the blank cases sheets in the mail. Sets of ten case sheets were mailed to each veterinarian during August and November 1994 and February and May 1995 with a reply paid envelope for return once completed. Case sheets recorded details of the signalment and anamnesis of the animal, the clinical signs noted, presumptive diagnosis, and details of treatment, management and prognosis as appropriate. Data from completed useable case sheets were entered into a specifically designed Microsoft Access (Microsoft Corporation) database using a standardised classification of clinical signs and diagnoses in use by the equine hospital at the University of Glasgow at the time. Disease diagnoses were subsequently classified into disease categories, largely relating to the body system affected, by the authors.

2.2.3 Comparison of BEVA and HRH Studies

For the present study, data listed in the 'British Equine Veterinary Association Survey of Equine Disease 1962-63' (COOK 1965) were manually entered into a spreadsheet in Microsoft Excel (Microsoft Corporation). Using the existing electronic database in Microsoft Access compiled during the HRH study, all case reports were reassigned to one of thirteen categories of disease as dictated in the BEVA study, in order that the datasets could be compared directly. Breeds of horse in the HRH study were grouped into 'racehorse', 'hunter', 'heavy horse' or 'pony' types, as used in the BEVA study. All cases described as being 'routine' such as pregnancy diagnosis and vaccinations were removed from the HRH study database, along with any repeated visits to the same animal to avoid replication. Other cases excluded from analysis were those reporting 'no abnormality detected', and cases from donkeys. Where no breed was recorded for a case in the HRH dataset, or where the breed was denoted as 'other' (a total of 9 cases out of 294, 3.1%), the case was assigned to the hunter category.

Following reclassification and preparation of the available data, comparisons of horse population data were performed using chi-square analyses in Minitab (Minitab Statistical Software Version 15). Crude period prevalence was calculated for each classification of disease in both studies, with confidence intervals for each result using an online adjusted-WALD asymmetric interval calculator (www.measuringusability.com). The prevalence of each disease classification within each horse type was also found. Where no overlap of confidence intervals occurred between paired proportions, the difference was considered to be significant. As appropriate, chi-square tests were used in Minitab version 15 Statistical Software Package to test the significance of changes in apparent disease prevalence between the two studies.

Table 2.1 All breeds of horse recorded in the HRH study were assigned to one of four 'types' as shown below. Horses recorded as 'other', or where breed was not recorded, were included in the Hunter type.

Breed Classification Group			
Racehorse	**Hunter**	**Pony**	**Heavy Horse**
Standardbred	Andalusian	Connemara	Clydesdale
Standardbred X	Anglo Arab	Connemara X	Clydesdale X
Thoroughbred	Appaloosa	Dales	Irish Draught
Thoroughbred X	Arab	Dartmoor	Shire
	Arab X	Exmoor	Shire X
	Cleveland Bay	Fell	
	Cleveland Bay X	Highland	
	Cob	Highland X	
	Cob X	New Forest	
	Eventer	Pony	
	Hack	Shetland	
	Haflinger	Shetland X	
	Hanoverian	Welsh	
	Hanoverian X	Welsh X	
	Hunter		
	Part Bred		
	Quarter Horse		
	Quarter Horse X		
	Show Jumper		
	Trakehner		
	Warmblood		
	Warmblood X		

Table 2.2 Example diagnoses from the BEVA study within the thirteen categories of disease.

Disease Classification	BEVA Study Example Diagnoses
Musculoskeletal	Fractures, Ringbone, Laminitis, Sandcracks
Alimentary	Helminthiasis, Colic, Hernia, Gingivitis
Urogenital	Anoestrus, Abortion, Retained Placenta
Respiratory	Strangles, Laryngeal Hemiplegia, Influenza
Skin	Dermatitis, Ringworm, Photosensitisation
Nervous	Head Shaking, Encephalitis, Facial Paralysis
Blood & Lymphatic	Azoturia, Lymphangitis, Septicaemia
Neoplasia	Melanoma, Carcinoma, Teratoma,
Cardiovascular	Pericarditis, Iliac Thrombosis
Systemic Disease	Tetanus, Brucellosis, Hyperthyroidism
Mammary Gland	Mastitis
Eye	Keratitis, Conjunctivitis, Cataract
Ear	Otitis

2.3 Results

2.3.1 BEVA Study Results Summary

The results of the BEVA study were published in 1965 (COOK 1965). Briefly, they were summarised as follows: in total 43,538 horses were registered with responding veterinary practices, and 17,268 cases of disease were reported. Musculoskeletal disease was the commonest category of disease reported (6588), followed by alimentary (4441), and urogenital (2434) conditions. The least commonly reported category was ear disease, with only 7 cases. The authors concluded that there was no seasonal or regional bias in terms of reported disease incidence.

2.3.2 HRH Study Results Summary

Some results from the HRH study have been published previously, but the disease data presented here have not (Mellor et al. 1999; Mellor et al. 2001). Briefly: seven hundred and sixty case sheets were mailed to the 19 sentinel veterinary practices which had agreed to take part. Twelve practices each returned a full complement of 40 case sheets, and 3 practices did not respond despite previously agreeing their involvement. Response rates were similar for all four seasons. Some case sheets were returned blank or unusable, and 20 case sheets were for repeated visits to individual horses. In total, 547 case sheets were returned and useable from 16 sentinel practices. One hundred and seventy eight routine consultations were documented, accounting for 33% of cases. Musculoskeletal disease was the most prevalent disease category, accounting for 28% of all cases seen, followed by alimentary disease (13%) and skin disease (9%) (see Fig. 2.2).

2.3.3 Results of Comparison of BEVA and HRH Studies

The geographical extent of the ‘North’ region of the BEVA study approximated that of the whole HRH study. The BEVA study used the number of horses registered with responding veterinary practices to estimate the size of the horse population at that time, whereas the HRH study used the number of horses owned by responding clients. While the derivation of these estimates differed, it was thought reasonable to use both to estimate changes in the

demographics of the equine population. When data from the North region of the BEVA study were compared with the HRH data, the representation of each horse type differed significantly between the studies (p<0.001). The BEVA study (North) reported that 60% of animals registered were ponies, 23% were hunters, 13% were racehorses and 4% were heavy horses. In contrast, horses owned in the HRH dataset comprised the following: 33% ponies, 31% racehorses, 28% hunters and 8% heavy horses.

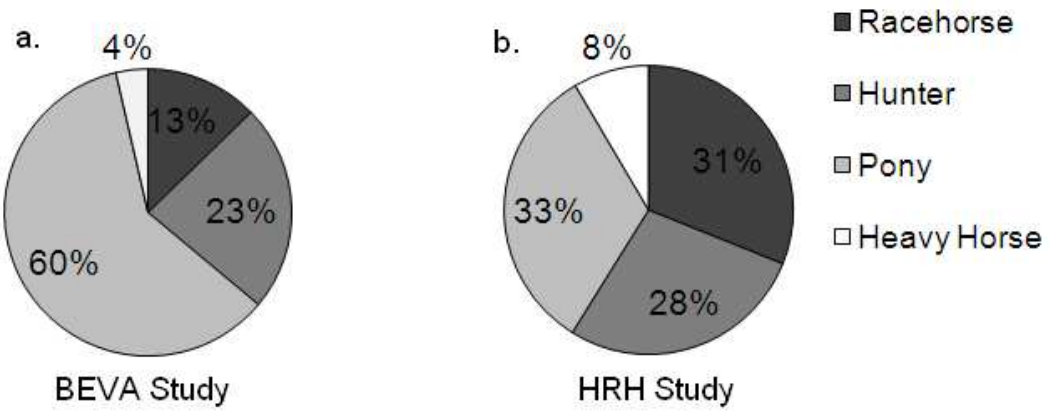

Figure 2.1 The composition of the equine population of northern Britain in 1965 and in 1997, where **a.** represents the BEVA (North) study (n=13,960) and **b.** the HRH study data (n=1252).

For each study, the proportions of each horse type seen by veterinarians were ranked. In both studies, hunters contributed most case reports followed by racehorses, ponies and finally heavy horses (see Table 2.3).

Table 2.3 Each horse type in the BEVA and HRH Studies ranked by their relative abundance in the 'population' at the time of each study, and by the relative proportion of veterinary case reports from each horse type.

Rank Position	BEVA Study		HRH Study	
	Relative Population Size	Contribution to Case Reports	Relative Population Size	Contribution to Case Reports
1	Ponies	Hunters	Ponies	Hunters
2	Hunters	Racehorses	Racehorses	Racehorses
3	Racehorses	Ponies	Hunters	Ponies
4	Heavy Horses	Heavy Horses	Heavy Horses	Heavy Horses

Table 2.4 The number of case reports of each disease category in each study, with their relative proportions.

Disease Category	BEVA Study		HRH Study	
	Number of Cases	%	Number of Cases	%
Musculoskeletal	6588	41.2	164	55.8
Alimentary	4441	27.8	39	13.3
Urogenital	1138	7.1	19	6.5
Respiratory	1776	11.1	22	7.5
Skin	935	5.9	26	8.8
Blood & Lymphatic	306	1.9	4	1.4
Neoplasia	226	1.4	4	1.4
Cardiovascular	185	1.2	0	-
Eye	149	0.9	3	1.0
Systemic	140	0.9	12	4.1
Nervous	54	0.3	0	-
Mammary Gland	27	0.2	1	0.3
Ear	7	0.0	0	-

Analysis of the six most prevalent disease classifications (with all other classifications amalgamated into a seventh category) revealed a significant alteration in the pattern of disease categories reported between the studies ($p<0.001$). Musculoskeletal disease formed the majority of cases seen in both the BEVA and HRH studies. Between the studies, the proportion of all diagnoses which reported musculoskeletal disease increased (41.2 ± 0.8% to 55.8 ± 5.7%). Systemic disease also increased in proportion from the BEVA to the HRH study (0.9 ± 0.1% to 4.1 ± 2.3%). Other significant changes comprised a fall in alimentary disease diagnosis proportion (27.8 ± 0.7% to 13.3 ± 3.9%), and a fall in respiratory disease diagnosis proportion (11.1 ± 0.5% to 7.5 ± 3%). No cases of cardiovascular, nervous or ear disease were reported in the HRH study. No other disease categories altered in proportion when all horse types were considered together.

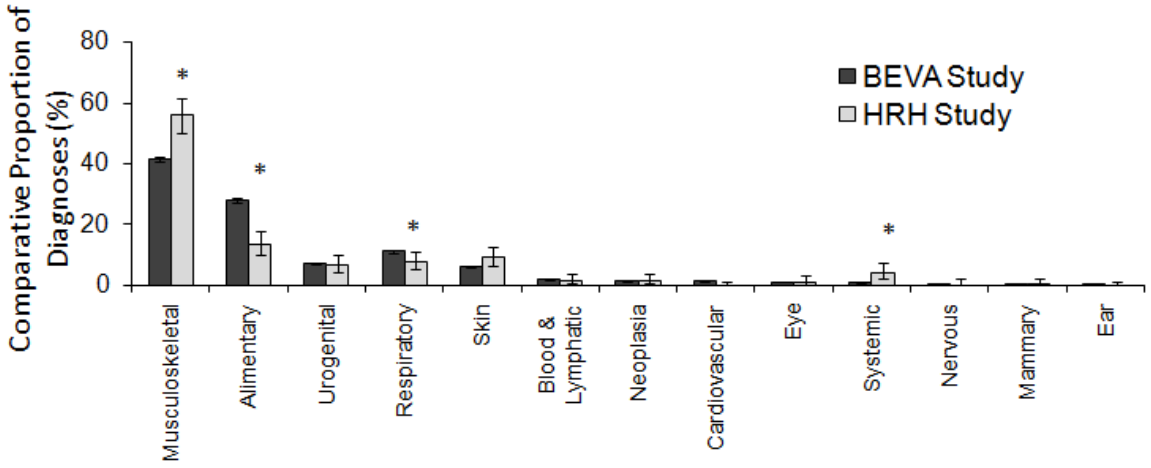

Figure 2.2 Comparison of the number of diagnoses of each disease category as a percentage of all diagnoses in each study. Error bars indicate 95% confidence intervals. (* indicates significance as defined by non-overlapping confidence intervals).

2.4 Discussion

In summary this study found that the demographics of the horse population of Scotland and northern England altered significantly during the period between the 1960s and 1990s, as did the reported prevalence of the disease categories featured. Ponies were the most abundant equine type in northern Britain in the BEVA study, whereas the HRH study showed a more even distribution of animals between the pony, hunter and racehorse categories. Despite ponies being the most abundant horse type in both studies, hunters were more frequently attended by veterinarians. The reporting of musculoskeletal and systemic diseases appeared to increase, while reports of alimentary and respiratory diseases became less common over the time period between studies.

These two studies represent the only two in recent times that have attempted to describe the disease profile of the general equine population of a significant part of the UK. Although the HRH study contains relatively few case records compared to the BEVA study, the data are valuable and make interesting comparisons in terms of major trends. Comparisons between the studies are only made at the disease category level as consistency of diagnosis below this level would make such comparisons subject to bias.

Currently, there exist two forms of 'surveillance' of the UK equine population, namely the National Equine Database (www.nedonline.co.uk), and the Animal Health Trust quarterly

disease reports (www.aht.org.uk/equine_disease.html). The National Equine Database (NED) Ltd is an organisation supported and guided by the British Equine Federation (BEF), which was set up in 2006 to allow collation, storage and supply of information on every equine born in the UK which has a passport. NED stores information on the name, age, gender, height, colour, pedigree and performance of each animal listed, and allows free access of the public to much of this information. NED could not be used in this analysis as it would have excluded those horses without passports, and no information on disease is recorded in the database.

The Animal Health Trust (AHT) in collaboration with the Department for Environment, Food and Rural Affairs (DEFRA) and the British Equine Veterinary Association (BEVA) began collating information on the occurrence of equine disease in the UK in 2004, and publishes a report on a quarterly basis. Disease incidence information is collected from various diagnostic laboratories and veterinary practices throughout the country. The AHT state that bias may be a feature of the data in these reports due to the differing motivations of owners in seeking veterinary attention. Although a useful resource, these reports do not assess the prevalence of non-infectious disease, nor do they give information about the location of the veterinary practices involved, and as such could not be used in this analysis.

There is a degree of inherent systematic error involved when horse breeds are grouped crudely into ‘types’. The racehorse and hunter type categories used in the BEVA study imply the nature of activity undertaken by an animal, rather than its phenotypic appearance or breed, and provide no middle ground for horses used in multiple pursuits. The main type of work undertaken by each horse in the HRH study is not known for the animals presenting to veterinarians, thus breed was used as a proxy for usage to enable reclassification, with the caveat that these groupings are rudimentary at best. The hunter category was the most prevalent among HRH cases (108/294, 36.7%) and contained the greatest diversity of breeds, and therefore was thought the most appropriate group in which to place horses whose breed was unknown.

The majority of disease categories did not appear to alter in their contribution to overall disease diagnoses between the BEVA and HRH studies, namely mammary, neoplastic, blood and lymphatic, cardiovascular, nervous, urogenital, skin, eye and ear diseases. However, for some of these categories no cases were recorded, therefore their ‘prevalence’ cannot be

commented upon with certainty. Musculoskeletal disease was the most frequently diagnosed category in both studies, followed by alimentary disease. Although the proportion of diagnosed systemic disease appeared to increase between the studies, only a small number of case records of this category were available, thus no conclusions can be reliably drawn from this result.

The most commonly occurring horse types in the 'population' of both studies were not the type most likely to receive veterinary attention. Excluding heavy horses which were consistently few in number, the makeup of the remainder of the equine population in the 1960s and 1990s differed significantly. Ponies were the most abundant of the four horse types at the time of the BEVA study, and hunters were the most abundant type during the HRH study. Despite these differences, a defined 'hierarchy' of horse types emerged when the numbers of case reports for each type were compared and this hierarchy was identical in both studies. In both studies, ponies were the most abundant horse type in the population, but they were only the third most likely type to receive veterinary attention. This discrepancy could reflect the relative disease resistance of ponies compared with other types of horse, differences in the intensity or type of exercise to which ponies are subjected, or potentially a neglect of their requirement for veterinary assistance in some circumstances. Hunters received the most veterinary attention, followed by racehorses, irrespective of their numbers in the population.

In the years following the BEVA study, the socioeconomic status of the average person in the UK improved. The proportion of the population falling within the age group thought most likely to participate in equestrian activities increased by 2.5% between 1981 and 1991 (Suggett 1999). During the 1990s inflation began to decline, the GDP stabilised, unemployment dropped, and an increase was seen in spending on recreation, probably as a function of increased disposable income (Suggett 1999). Owning a horse therefore became financially accessible to many more people, and the proportion of first-time horse owners presumably increased. First-generation horse owners cannot benefit from the 'instinct' and knowledge of experienced relatives. Despite the plethora of literature available on horse care, a novice owner will make different judgement calls about husbandry and when to call for veterinary attention, when compared with a more experienced horse-person. It is possible that both an increase in available information, and a lack of horse care experience, may have contributed to the apparent changes in disease prevalence seen in this study.

Not surprisingly, musculoskeletal disease remained the most frequently observed category of disease in horses. Its dominance probably stems from a combination of the fragile, unique anatomy of the equine locomotor system, and the intensity and diversity of work to which these animals are put.

In the thirty years that elapsed between the BEVA and HRH studies significant advances were made in veterinary research across many fields. The precise nutritional requirements of horses of every size and activity level were deduced and a plethora of scientifically formulated feedstuffs became available. As Harris opined in 1999, it is probable that 'almost every permutation of feeding regimen could be found somewhere in the UK' (Harris 1999). Diet could affect the prevalence of alimentary disease directly by being a cause of colic or gastric ulceration, for example (Harris 1998; Durham 2009). Feeding practices have also been identified as important in the genesis of disease (Harris 1999; Luthersson et al. 2009). The relative reduction in cases of alimentary disease in the HRH study may in part reflect the evolution of improved, scientifically derived feeds and feeding regimens.

Novel anthelmintic agents to supersede phenothiazine were discovered after the 1960s, such as the benzimidazoles and the organophosphates (Lyons *et al.* 1999). Discovery of the efficacy of levamisole, pyrantel and the macrocyclic lactones in the 1970s and 1980s improved the control of helminths in the UK dramatically, and intensive research into mechanisms of parasite resistance allowed worming strategies and pasture management techniques to be found which could reliably reduce worm burdens (Lyons *et al.* 1999). Although Mellor *et al.* reported that owners surveyed in the HRH study were worming their horses at suboptimal frequencies, due to the use of more efficacious products and improved worming regimens it is likely that worm control in the 1990s was greatly superior to the 1960s (Mellor *et al.* 2001). The apparent reduction in reporting of alimentary disease may also be linked to the increasing tendency of horse owners to utilise the services of Equine Dental Technicians in the event of supposed alimentary disease, rather than the veterinarian.

Vaccination against infectious agents such as Influenza A are now widespread. Influenza vaccines were first produced in the 1960s as inactivated whole virus or viral surface antigens (Minke *et al.* 2004). The protection offered by these early vaccines correlated well with the

amount of circulating antibody to the haemagglutinin surface glycoprotein, but the large outbreak of influenza in 1979 proved that the protection offered by these vaccines was suboptimal (Minke *et al.* 2004). Current vaccines have been manufactured which offer greater efficacy, with new adjuvants for improved potency. Hotchkiss *et al.* reported in 2007 that nearly 56% of horses in Great Britain were vaccinated against influenza and tetanus, with only 9.2% of horses never being vaccinated (Hotchkiss *et al.* 2007). Mellor *et al.* reported a population mean rate of vaccination of 0.8 ± 0.41 doses/horse/year of horses in Scotland and northern England in the HRH study, and that 17% of horses were never vaccinated (Mellor *et al.* 2001). This rate falls below the recommended vaccination rate of 1 dose/horse/year (for influenza vaccines). The vaccine coverage of horses in the 1960s is not known, but it is thought that the rate of dosing and use of products of improved efficacy in the HRH study would likely constitute superior protection from influenza. The respiratory disease category, containing influenza as a possible diagnosis, reduced in its contribution to disease diagnoses between the BEVA and HRH studies. It is possible that this reduction could be linked to improved protection from influenza by vaccination.

In conclusion, these studies have provided the most comprehensive description of the equine population of Great Britain in terms of its composition and disease experience. Given the size of the equine population and its connectedness with human, domestic and wild animal populations, the importance of up to date and accurate information on the health status of such a significant biomass cannot be overstated.

CHAPTER 3 - Preliminary Findings of a Case-Control Study of Zoonotic Enteric Diseases in Rural Western Kenya

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CHAPTER 3 - Preliminary Findings of a Case-Control Study of Zoonotic Enteric Diseases in Rural Western Kenya

Abstract

Diarrhoea is the second most common cause of death of children under 5 years of age in the developing world, and accounts for more child deaths than measles, malaria and HIV combined. An important cause of such enteric disturbances is infection with pathogenic organisms, many of which may be acquired through zoonotic transmission from domestic animal species. Detailed epidemiological information regarding the causative agents implicated in diarrhoeic disease in western Kenya is lacking. The Kenyan Medical Research Institute (KEMRI) in collaboration with the Centres for Disease Control and Prevention (CDC) began conducting a case-control study of diarrhoeic children in western Kenya in 2008.

Results

Ninety seven and a half percent of compounds housed chickens, 75.9% housed dogs and 74.7% kept cattle, other species were less common. Two point four percent of pooled animal faecal samples were classified as diarrhoeic faeces, 66.7% of which were from young animals. The following pathogens were found in animal faecal samples at the stated prevalences (all animal species sample results combined); *E. coli* 99.3%, *Salmonella enterica* 6.6%, *Campylobacter jejuni* 1.8%, *Campylobacter coli* 0.7%, *Salmonella typhi* 0.2%, *Giardia* spp. 10.2% and *Cryptosporidium* spp. 2.5%. In stool samples from children, the following pathogens were found to be marginally more prevalent in cases versus than controls; *C. jejuni*, *C. coli*, *S. enterica*, and *Cryptosporidium*. *Giardia* spp. were more prevalent among control children than cases. No statistically significant associations were found between the presence of any of the identified pathogens in the animal population of a compound with its presence in the child samples of the same compound.

3.1 Introduction

Diseases of animal origin are prevalent throughout the world. Enteric disturbances such as diarrhoea are a very common manifestation of such diseases. The Centres for Disease Control and Prevention (CDC) estimate that 75% of recently emerging infectious diseases of humans originated from animal sources some time in the past(www.cdc.gov 2010). Developing parts of the world often suffer high rates of such infections due in part to a lack of safe drinking water, poor hygiene and poor sanitation, coupled with living in close proximity to domestic animal species. Young children are particularly susceptible to dehydration from diarrhoea due to their relatively high metabolic rate: therefore diarrhoea is a leading cause of death in children under 5 years of age, worldwide (www.who.int 2010). In Kenya it is estimated that 16% of the deaths of children under the age of five years are due to diarrhoea, but the precise combination of enteric pathogens implicated is unknown (www.who.int 2010). In 2008 a large scale case-control study by the CDC, in collaboration with the Kenyan Medical Research Institute (KEMRI), began in order to identify those pathogens present in stool samples from diarrhoeic young children in western Kenya, and the lifestyle factors associated with infection. In order to study the contribution of zoonotic enteric pathogens to this disease profile, the Zoonotic Enteric Diseases Study (ZED) was designed to run alongside the child study (GEMS). Sample collection and analysis are ongoing at the time of writing. The aims of the ZED study are to identify which potentially zoonotic pathogens are implicated in the burden of childhood diarrhoea within the test area, and to generate recommendations for practical interventions which could reduce the rate of infection in the future. This paper reports on some of the preliminary results from the testing of both animal and child faecal samples. For the purposes of this study, the term ‘zoonotic’ is taken to mean those infectious diseases, harboured by animals, which are considered to be a threat to humans in the present day, as opposed to those pathogens which became infectious to humans in the past but are no longer acquired from animal sources.

3.2 Materials and Methods

3.2.1 Global Enterics Multi-Centre Study (GEMS)

The Global Enterics Multi-Centre Study (GEMS) called 'Diarrhoeic Disease in Infants and Young Children in Developing Countries' commenced in 2008. Children under five years of age suffering from moderate to severe diarrhoea, who received medical attention in the Gem and Asembo Demographic Surveillance System Area of rural western Kenya were invited to become enrolled as 'cases' in the GEMS study. The guardian of each case child completed a questionnaire covering subjects such as the child's age and sex, hygiene practices, water sources, water storage and handling, and whether domestic animals were kept in or near the home. A faecal sample was taken from each child to be processed at the KEMRI/CDC laboratories at Kisian. Another, non-diarrhoeic, child from the same compound which was matched for age and sex with the case child, was enrolled as a control. The same questionnaire and faecal sample processes were used with case and control children. Enrolment and sample testing procedures are ongoing at the time of writing.

3.2.2 Zoonotic Enteric Diseases Study (ZED)

The caretakers of case and control children in the GEMS study which reported the presence of domestic animals on their compound were approached to be enrolled on the ZED study. Each enrolled compound was visited within 3 days of a child being included into GEMS, and two questionnaires were administered. The first questionnaire was administered to the caretaker of the child in question, and covered current and recent animal ownership, management, handling practices, and risk factors for exposure to animals and their environment. The second questionnaire was administered to the head of each compound, in order to ascertain the numbers, age groups and species of domestic animals housed on the compound. Faecal samples were collected from all or a proportion of the domestic animals present, depending on population size. For large animals, faecal samples were collected per rectum using a gloved hand or digit. For small animals such as puppies and kittens, sampling was achieved by inserting a sterile cotton swab into the rectum. Poultry were confined on a thick plastic sheet overnight and the droppings collected the following morning. All faecal samples were pooled by animal species and age group ('young' i.e. unweaned, 'adult' i.e. weaned, or 'mixed ages'),

with a maximum of 5 animal samples per pool, and a maximum of 2 pools per species/age group, with each pool being thoroughly mixed using a sterile spatula. Three rectal or cloacal swabs were also collected per animal, two of which were placed into Cary Blair medium, and one into buffered glycerol saline (BGS). All samples were appropriately labelled and stored on ice for transport to the laboratory. The processes involved in the ZED study are summarised below (see Figure 3.1).

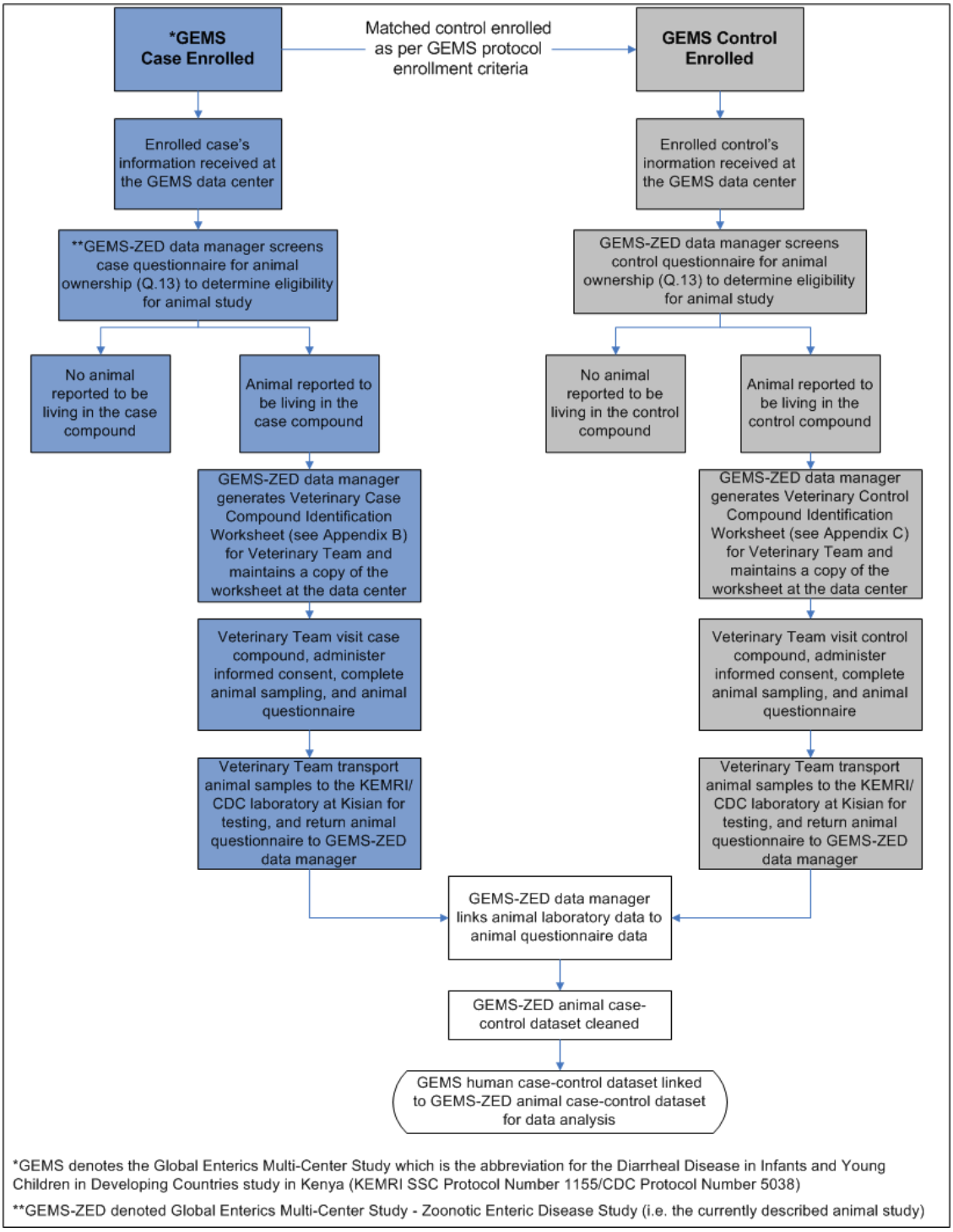

Figure 3.1 Flowchart for the Zoonotic Enteric Diseases Study.

3.2.3 Sample Testing

Faecal samples from the case and control children were subjected to various laboratory procedures to identify a range of enteric pathogens including *Salmonellae, Shigella* spp., *Cryptosporidium* spp., *Giardia* spp., *Norovirus, E. coli* and *Campylobacter*. Procedures implemented included Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR), bacterial culture on selective media, and Enzyme-Linked Immunoassays (ELISAs). Typing and sub-species determination of pathogens recovered was completed at KEMRI laboratories if appropriate equipment and skills were available: alternatively samples were shipped to the CDC in Atlanta, US. Animal faecal samples were also cultured to identify bacterial species using the same methods as the GEMS study, and were subjected to ELISA using commercial immunoassay kits to detect *Giardia* spp. or *Cryptosporidium* spp. (Wampole Giardia II, TechLabs, Wampole Cryptosporidium II, TechLabs). Testing for the detection and sub-typing of the following pathogens is ongoing; *E. coli, Salmonella, Shigella* spp., *Campylobacter, Giardia* spp., *Cryptosporidium* spp., *Rotavirus.* Figure 3.2 illustrates a simplified version of the processes for pathogen detection used in the ZED study.

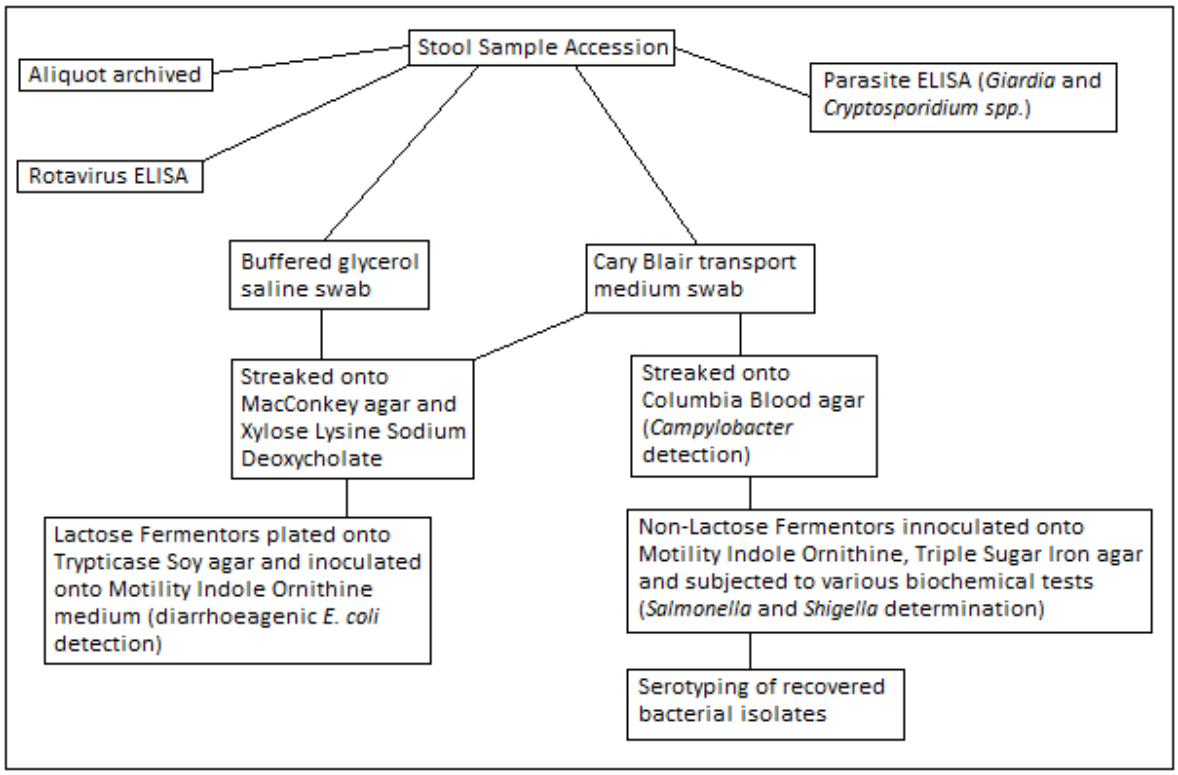

Figure 3.2 Simplified flow chart depicting the stages of faecal sample processing to identify zoonotic enteric pathogens in the ZED study. Detailed biochemical testing procedures, sub-typing and further speciation methods are beyond the scope of this paper.

Data collected in the ZED study were computerised using optically scannable forms and Teleform software, and stored in a Microsoft Access Database, which was subsequently exported into Microsoft Excel for analysis. Where possible, associations between variables were investigated using Fisher's Exact Tests.

3.3 Results

3.3.1 Animal Faecal Samples

In total 511 pooled animal faecal samples were collected and stored at KEMRI (Kisian, western Kenya) between the 27th November 2009 and 1st February 2010. To date, 455 samples have been subjected to bacterial culture, and 403 to enzyme-linked immunoassay (ELISA) for protozoa (*Giardia* spp. and *Cryptosporidium* spp.). Between 1 and 9 animals contributed to each pool (mean 3.1, median 5). The species and age distribution of all pools collected is shown in Table 3.1.

Table 3.1 Age classification and species of all faecal sample pools in GEMS-ZED database, n=511.

	Number (%) of animal faecal sample pools of each age group and species			
	Young	**Adult**	**Mixed**	**Total**
Cattle	77 (20.5)	30 (29.4)	1 (3.0)	108 (21.1)
Sheep	34 (9.0)	9 (8.8)	1 (3.0)	44 (8.6)
Goats	68 (18.1)	19 (18.6)	1 (3.0)	88 (17.2)
Chickens	98 (26.1)	39 (38.2)	27 (81.8)	164 (32.1)
Dogs	57 (15.2)	0 (0.0)	0 (0.0)	57 (11.2)
Cats	32 (8.5)	3 (2.9)	0 (0.0)	35 (6.8)
Donkeys	9 (2.4)	2 (2.0)	0 (0.0)	11 (2.2)
Ducks	1 (0.3)	0 (0.0)	3 (9.1)	4 (0.8)
All Animals	376 (100.0)	102 (100.0)	33 (100.0)	511 (100.0)

Twelve (2.4%) pools were classified as diarrhoeic, 2 (0.4%) contained blood, 1 (0.2%) contained pus and 3 (0.6%) contained mucus (see Table 3.2). Of those pools which were diarrhoeic, 8 (66.7%) were classified as young (unweaned), 2 (16.7%) as adult (weaned), and 2 (16.7%) contained samples from animals of mixed ages.

Table 3.2 Number (% within each species) of faecal specimen pools which were classified as diarrhoeic, non-diarrhoeic, or containing pus, mucus or blood.

	Total Pools	**Not diarrhoea**	**Diarrhoea**	**Blood Present**	**Pus Present**	**Mucus Present**
Cattle	108	103 (95.4)	5 (4.6)	1 (0.9)	0 (0.0)	0 (0.0)
Goat	86	80 (93.0)	6 (7.0)	1 (1.2)	0 (0.0)	3 (3.5)
Sheep	43	43 (100.0)	0 (0.0)	0 (0.0)	1 (2.3)	0 (0.0)
Chicken	164	163 (99.4)	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)
Dog	57	57 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cat	35	35 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Donkey	11	11 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Duck	4	4 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

E. coli was the pathogen most frequently detected in animal faeces, with an overall prevalence of 99.3% (see Table 3.3). Other bacterial pathogens were found to have a low prevalence

across all species sampled, with the exception of *Salmonella enterica* in chickens (13.2%) and cats (11.8%). Of those protozoal genera identified using commercial ELISA kits, *Giardia* spp. were most prevalent, with ovine and canine species having a *Giardia* prevalence of 25.7% and 32.0%, respectively.

Table 3.3 Results of animal faecal sample tests by either bacterial culture, or ELISA.

	Positive test results/Total tests run (%)								
	Sheep	**Cattle**	**Goats**	**Chickens**	**Dogs**	**Cats**	**Donkeys**	**Ducks**	**All Species**
C. jejuni	0/37 (0.0)	0/99 (0.0)	0/70 (0.0)	8/152 (5.3)	0/50 (0.0)	0/34 (0.0)	0/10 (0.0)	0/3 (0.0)	8/455 (1.8)
C. coli	0/37 (0.0)	0/99 (0.0)	2/70 (2.9)	1/152 (0.7)	0/50 (0.0)	0/34 (0.0)	0/10 (0.0)	0/3 (0.0)	3/455 (0.7)
S. typhi	0/37 (0.0)	0/99 (0.0)	0/70 (0.0)	1/152 (0.7)	0/50 (0.0)	0/34 (0.0)	0/10 (0.0)	0/3 (0.0)	1/455 (0.2)
S. enterica	1/37 (2.7)	0/99 (0.0)	1/70 (1.4)	20/152 (13.2)	4/50 (8.0)	4/34 (11.8)	0/10 (0.0)	0/3 (0.0)	30/455 (6.6)
E. coli	37/37 (100.0)	96/99 (97.0)	70/70 (100.0)	152/152 (100.0)	50/50 (100.0)	34/34 (100.0)	10/10 (100.0)	3/3 (100)	452/455 (99.3)
***Giardia* spp.**	9/35 (25.7)	6/95 (6.3)	5/67 (7.5)	5/143 (3.5)	16/50 (32.0)	0/34 (0.0)	0/10 (0.0)	0/3 (0.0)	41/403 (10.2)
***Cryptosp-oridium* spp.**	0/35 (0.0)	0/95 (0.0)	0/67 (0.0)	7/143 (4.9)	3/50 (6.0)	0/34 (0.0)	0/10 (0.0)	0/3 (0.0)	10/403 (2.5)

3.3.2 Child Stool Sample Results

In total to date, 127 children have been enrolled onto the GEMS study, of which 50 are cases and 77 are controls. Of those children, 79 have also been enrolled onto the ZED study, of which 39 are cases and 40 controls. The average age of case children is 16.2 months (range 2-48 months), and 53.8% (21/39) are male. The average age of control children is 16.4 months (range 0-48 months) and 52.5% (21/40) are male. The animals present at compounds where case or control children reside is given in table 3.4 below.

Table 3.4 Reported number (%) of compounds to house each animal species.

	Animal species present at residential compounds							
	Cattle (%)	**Sheep (%)**	**Goats (%)**	**Dogs (%)**	**Cats (%)**	**Fowl (%)**	**Donkeys (%)**	**No animals (%)**
Cases n=39	30 (76.9)	15 (38.5)	29 (74.4)	27 (69.2)	23 (59.0)	37 (94.9)	3 (7.7)	1 (2.6)
Controls n=40	29 (72.5)	16 (40.0)	24 (60.0)	33 (82.5)	33 (82.5)	40 (100.0)	1 (2.5)	0 (0.0)
Total n=79	59 (74.7)	31 (39.2)	53 (67.1)	60 (75.9)	56 (70.9)	77 (97.5)	4 (5.1)	1 (1.3)

E. coli was the most frequently isolated bacterial species in the child samples (see Table 3.5). The prevalence of *C. jejuni*, *C. coli*, *S. typhi* and *E. coli* were similar or slightly higher in case versus control children. *S. enterica* was more prevalent among cases than controls, and *Giardia* spp. were found to be more prevalent among controls than cases.

Table 3.5 Prevalence of select pathogens found in child faecal samples.

	Number (%) of faecal samples positive per pathogen						
	***C. jejuni* (%)**	***C. coli* (%)**	***S. typhi* (%)**	***S. enterica* (%)**	***E. coli* (%)**	***Giardia* spp. (%)**	***Cryptosporidium* spp. (%)**
Cases n=39	5 (12.8)	1 (2.6)	0 (0.0)	4 (10.3)	38 (97.4)	7 (18.0)	6 (15.4)
Controls n=40	4 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	39 (97.5)	12 (30.0)	2 (5.0)
Total n=79	9 (11.4)	1 (1.3)	0 (0.0)	4 (5.1)	77 (97.5)	19 (24.1)	8 (10.1)

Table 3.6 shows that the majority of samples were negative for bacterial and protozoal enteropathogens (excluding *E. coli*) in both the child and animal samples tested. *E. coli* was equally highly prevalent among the child and animal samples of cases and controls.

Table 3.6 Compound allocation into pathogen present (+), absent (-) or missing (m) in sampled children, and animals. (*Crypto.* spp. = *Cryptosporidium species*).

			Animal Sample Results																				
			C. jejuni			*C. coli*			*S. typhi*			*S. enterica*			*E. coli*			*Giardia* spp.			*Crypto.* spp.		
			-	+	m	-	+	m	-	+	m	-	+	m	-	+	m	-	+	m	-	+	m
Child Samples	Cases n=39	-	31	2	0	37	0	0	38	0	0	28	6	0	0	0	0	17	11	0	27	2	0
		+	4	1	0	1	0	0	0	0	0	4	0	0	1	37	0	4	3	0	6	0	0
		m	1	0	0	1	0	0	1	0	0	1	0	0	0	1	0	4	0	0	4	0	0
	Controls n=40	-	33	2	0	39	0	0	38	1	0	27	12	0	0	0	0	17	7	0	33	1	0
		+	4	0	0	0	0	0	0	0	0	0	0	0	1	38	0	7	5	0	2	0	0
		m	0	1	0	1	0	0	1	0	0	0	1	0	0	1	0	3	1	0	2	2	0
All Samples n=79		-	64	4	0	76	0	0	76	1	0	55	18	0	0	0	0	34	18	0	60	3	0
		+	8	1	0	1	0	0	0	0	0	4	0	0	2	75	0	11	8	0	8	0	0
		m	1	1	0	2	0	0	2	0	0	1	1	0	0	2	0	7	1	0	6	2	0

The detected presence of any of the above pathogens within the animal population of a compound was found not to be statistically significantly associated with the presence of that pathogen in the child stool sample of the same compound (p>0.05). Age group and pool size were not found to be predictors of detection of any of the above pathogens. A pool size of 2 (i.e. 2 animal samples contributing to one pool) was found to be most likely to test positive for at least one enteropathogen, in comparison to other pool sizes (see Table 3.7). Twenty percent of pools were found to contain at least one of the target pathogens (excluding *E. coli*).

Table 3.7 The number of animal faecal samples contributing to a pool ('pool size'), versus the number (and proportion) of those pools found to contain at least one of the following pathogens; *Campylobacter jejuni, Campylobacter coli, Salmonella typhi, Salmonella enterica, Giardia* or *Cryptosporidium*.

Pool Size	Number (%) of pools with at least one positive pathogen result (excluding *E. coli*)	Total number of pools subjected to both culture and ELISA
1	15 (19.0)	79
2	21 (28.0)	75
3	10 (20.4)	49
4	6 (12.5)	48
5	28 (19.0)	143
Over 5	1 (25.0)	4
All Pool Sizes	81 (20.4)	398

Animal and child sample results which were available for analysis were grouped according to the calendar year in which the sample was received at the laboratory. Six full months of results for GEMS (November to April) and seven full months of ZED results (December to June) were available for analysis (see Figures 3.3 and 3.4). Preliminary test results for six selected pathogens revealed that most positive test results were recorded for those samples received in February in both animal and child faecal samples.

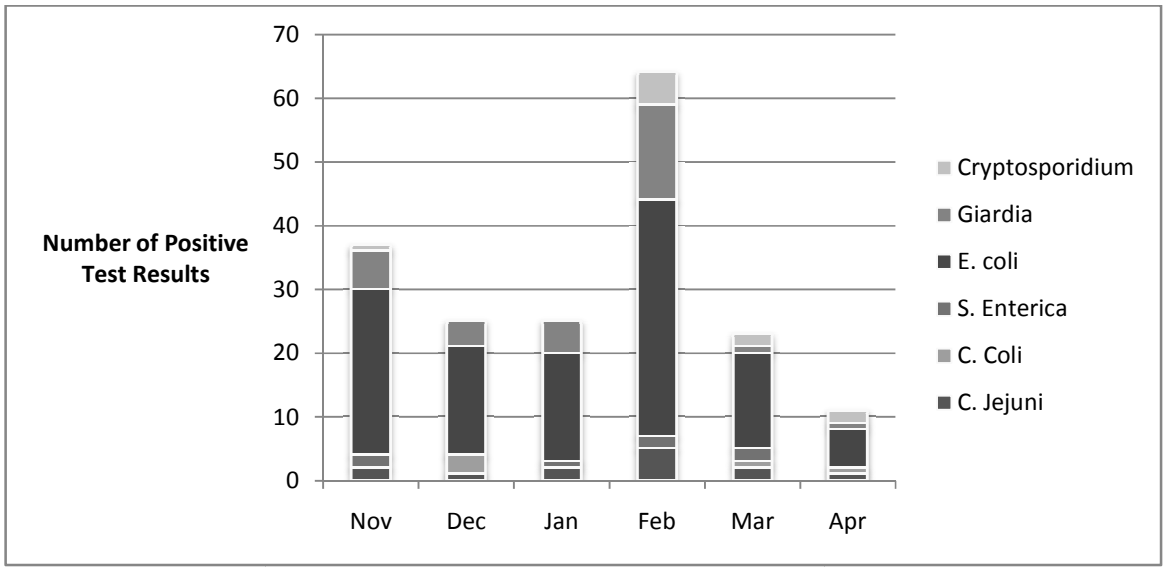

Figure 3.3 Temporal 'pattern' of positive test results (by bacterial culture or ELISA) in child stool samples by month of enrolment onto GEMS.

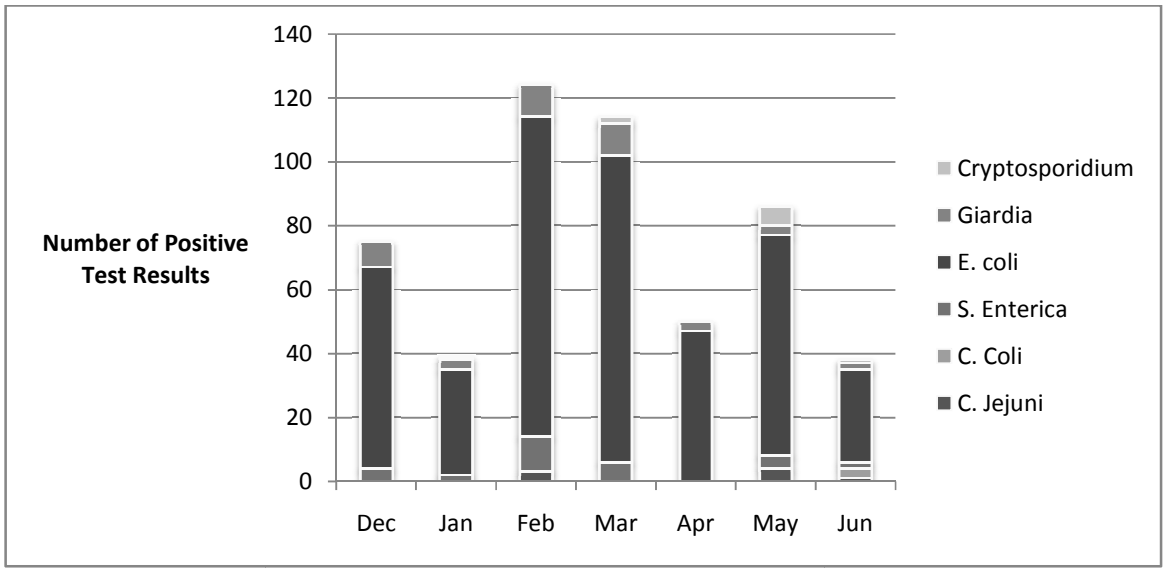

Figure 3.4 Temporal 'pattern' of positive test results (by bacterial culture or ELISA) in animal faecal samples by month of enrolment onto GEMS-ZED.

3.4 Discussion

The importance of the contribution of zoonotic enteric pathogens to the disease burden of children in the developing world should not be underestimated. The WHO estimates that 2.5 billion cases of childhood diarrhoea occur annually, resulting in 1.5 million child deaths, of

which a large proportion are due to avoidable enteric infections . Diarrhoea is responsible for 16% of the deaths of children under 5 years old in Kenya, the second most common cause of death after pneumonia (www.who.int 2010). From initial survey results from the GEMS study it was found that in the rural area being sampled, 99% of caretakers of young children reported the presence of domestic animals in or near the home. This figure excluded dogs, thus the true prevalence of animal ownership on residential compounds is likely to approach 100%. There is much evidence to support the role of zoonotic pathogens in human disease, but the prevalence of those pathogens likely to be implicated in rural western Kenya is yet to be fully elucidated (Atabay and Corry 1998; Nataro and Kaper 1998; Adak et al. 2002; Caccio et al. 2005; Hunter and Thompson 2005; Thompson et al. 2005; Gatei et al. 2006; Stirling et al. 2008). Exposure of children to zoonotic pathogenic organisms could occur through close contact, handling, sharing of environments, and the consumption of undercooked eggs, other foods of animal origin or of unpasteurised milk, or any other food contaminated with animal faeces (Atabay and Corry 1998; Poppe et al. 1998; Graham et al. 2000; Palombo 2002; Sanchez et al. 2002; Kariuki et al. 2006; Stirling et al. 2008; Bank-Wolf et al. 2010). The likelihood of mortality resulting from diarrhoea is increased by failure to instigate effective hygiene and sanitation practices, a lack of safe water sources, and failure to provide effective medical care. Up to 61% of childhood diarrhoea cases in the developing world do not receive adequate treatment in the form of oral rehydration therapy, which in many cases could prove life-saving (www.who.int 2010).

The prevalence of enteropathogens in the domestic animal populations of rural western Kenya has not previously been investigated using the methods employed in the GEMS-ZED study. Prevalence of the enteropathogens of children and domestic animals included in this preliminary report cannot be directly compared to figures obtained from other parts of the developing world or to older studies, due to a paucity of data in this field, and due to the small sample sizes available for analysis here.

The following pathogens were found to be more prevalent among case than control children; *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella enterica*, and *Cryptosporidium* spp. However, the difference in prevalence of these pathogens between case and control children was not found to be significant. Of these pathogens, the domestic animal species which was found to harbour the target pathogen with the greatest frequency was as follows: *C. jejuni* -

chickens, *C. coli* - goats, *S. enterica* - chickens, and *Cryptosporidium* spp. - dogs. Although each of these pathogens was found at a higher prevalence in case than control children, it is not possible to ascribe a causal link between diarrhoea and pathogen presence at this stage, or say with any certainty that children were infected through contact with those animal species commonly harbouring the pathogen. Chickens were found to be abundant in the compounds studied: indeed 97.5% of all compounds reported the presence of chickens, a greater proportion than reported the presence of any other animal species. Chickens have been found to harbour potentially zoonotic pathogens in a number of previous studies, and this study has shown that chickens are carriers of many of the pathogens detected thus far (Atabay and Corry 1998; Graham et al. 2000; Sanchez et al. 2002). Due to their abundance in the domestic setting and their tendency to harbour certain enteropathogens at a high prevalence, chickens may be found to be a significant risk factor for childhood diarrhoea.

Twelve animal faecal sample pools contained diarrhoeic faeces (2.4%). Animals were not excluded from the sampling procedure if they were found to have diarrhoea, nor were they specifically sought out, but samples from diarrhoeic animals were pooled separately from non-diarrhoeic samples. This number is too small to allow significant associations to be found between the presence of animal diarrhoea and the recovery of any particular enteropathogen. However, other studies have failed to find any association between animal diarrhoea and the presence of a known enteropathogen (De Rycke *et al.* 1986). The majority (66.7%) of diarrhoeic animal samples came from young animals. Potentially animals suffering from diarrhoea are more likely to transmit infectious zoonotic enteropathogens to susceptible children, although this hypothesis could not be tested using the small sample sizes in this analysis. If this is the case, it seems likely that a greater risk to children is posed by the presence of young animals than adult animals, as they more commonly suffer diarrhoea, which may be of an infectious nature.

Giardia spp. was found to be more prevalent among control children than case children in these preliminary data. This finding was found again after analysis of the GEMS-ZED database at a later date than was possible for the production of this paper (Omore *et al.* 2010). A significantly higher prevalence among control children may suggest that *Giardia* spp. are acting as a protective factor against childhood diarrhoea through unknown mechanisms. To the authors knowledge this finding has not been previously reported. The presence of dogs on

the compound is reported more commonly by caretakers of control children than case children, and dogs were found in this study to harbour *Giardia* spp. with a higher prevalence than any other animal species (32.0%). The possible link between domestic dog presence, *Giardia* spp., and an absence of diarrhoea in children warrants further investigation.

A temporal (annual or biannual) pattern of detection of some enteropathogens or of childhood bacteraemia has been previously reported, often coinciding with the rainy seasons of tropical and sub-tropical regions (Graham et al. 2000; Brent et al. 2006; Kariuki et al. 2006; Gordon et al. 2008). A peak of pathogen detection was seen in the animal and child samples submitted around February in this study. In Kenya, the long rainy season occurs from March to May, thus February could be thought of as a 'dry' month. High pathogen prevalence in child and animal faecal samples at this time could relate to a restricted water supply owing to the lack of rain, which leads available water sources to be overused and more likely contaminated. This explanation differs from those offered for peaks of pathogen detection during rainy seasons, which often relate to contamination due to flooding (Brent et al. 2006; Kariuki et al. 2006; Gordon et al. 2008). Small numbers of test results were available for analysis for the purposes of this report, and less than one year of sample submissions could be analysed, thus determination of any temporal pattern in positive results is tentative but will be studied in detail at the conclusion of the GEMS-ZED study.

To determine that the route of infection for a diarrhoeic child was zoonotic, the pathogen(s) implicated must be characterised using sub-typing to a certain level effectively to exclude its possible acquisition from alternative sources, i.e. the same species/sub-type/serotype of pathogen must be recovered from both the child and the animal in question (Nataro and Kaper 1998; Adesiyun et al. 2001; De Grazia S. et al. 2007; Smith et al. 2007; Koopmans 2008; Xiao and Fayer 2008; Bank-Wolf et al. 2010). Even when this information is known, it is not possible to be certain that zoonotic transmission occurred, as the direction of transfer may have been anthroponotic, for example. However, in these circumstances, zoonotic transfer is usually inferred. To allow identification of those pathogens and the animal species involved in possible zoonotic transfer to children, the GEMS-ZED study aims to characterise all of the pathogens involved using various methods. Pathogens other than those discussed in this paper are also being investigated for their role in childhood diarrhoea, e.g. *Rotavirus, Norovirus, Shigella* spp. This testing was not complete at the time of writing.

Some of the pathogens featured in the GEMS-ZED study are not shed from their host in a constant, predictable manner. For example *Giardia* spp. are often shed in a cyclically, therefore multiple pooled faecal samples are required for reliable diagnosis by faecal cyst detection methods. Although animal faecal samples in the GEMS-ZED study were pooled to include up to 9 animal samples per pool, some pools contained very few samples, therefore false negative results may have occurred.

Children in rural western Kenya often live in close proximity with domestic animals, thus are likely to be exposed repeatedly to pathogenic organisms harboured by those species. This exposure may confer a degree of immunity to the child, and thus enteric disease as a result of infection with those pathogens may become less likely. Additionally, testing of animal and child faecal samples may not detect recent infections, as there will be a delay infection and shedding. This may alter the supposed prevalence of such pathogens to a degree. For these reasons, detection of zoonotic enteric pathogens in animal species and children in the GEMS-ZED study will need to be interpreted with care.

Although zoonotic transfer is often inferred when a specific type of pathogen is detected in animal and human samples, it may be the case that person to person transfer occurred, with subsequent anthroponotic transfer. Indeed, person to person transfer could have led to a large proportion of the child infections seen thus far in the GEMS-ZED data, which would make study of the animal population less important. However, this theory cannot be proven at the present time, but must be taken into account in the final analysis of this data.

The GEMS-ZED study aims to identify those enteropathogens implicated in childhood diarrhoea, the domestic animal species from which they may derive, and to generate advice on practical interventions to avoid further infections. This preliminary report suffers from a lack of statistical power due to small sample sizes, and thus there is limited capacity to identify significant associations. However, it does suggest that interesting trends in the temporal pattern of diagnosis, in the possible protective effect of *Giardia* spp. infection, or domestic carnivore presence, may be emerging from these data, and are worthy of further study.

General Conclusions

Project 1

This study proved that the compound K11777, an inhibitor of cathepsin L-like cysteine proteases, has the ability to alter the incidence of spontaneous Ca^{2+} 'waves' in isolated adult rat cardiomyocytes which have previously been subjected to *T. b. brucei* supernatant. This result suggests that the increase in wave production seen due to the presence of *T. b. brucei* parasites or their supernatant, is due to parasitic secretion/excretion of a cathepsin L-like cysteine protease. It was not possible to prove the involvement of PAR 2 through the use of SLIGRL-NH_2 in this project.

Accurately pinpointing the pathways and chemical signals involved in the cardiac pathologies of Trypanosomiasis may have important implications. It is possible that there is overlap in the mechanisms by which the parasite causes Ca^{2+} 'waves' (and therefore arrhythmias), and penetrates the blood-brain barrier leading to neurological derangement. If this is the case, a drug which is able to abrogate the first effect thereby abolishing cardiac disturbances, may also prevent the second effect, thus the major cause of the morbidity and mortality in Human African Trypanosomiasis could potentially be prevented. Future research should focus on establishing which PAR(s) (if any) are involved in the instigation of intracellular Ca^{2+} 'waves', through more extensive use of both PAR-APs and inhibitory molecules. The hypothesis of protease activation of the iRAS pathway should also be investigated in further studies.

Project 2

As was hypothesised, the profile of equine disease in the UK (as defined by the relative proportions of disease categories reported to veterinarians) was found to have significantly altered in the time between the BEVA and HRH studies in this project. There was also some evidence that the composition of the equine population of northern UK altered over the test period, in terms of the types of breeds present.

The non-racing equine population of the UK is largely neglected in terms of epidemiological and demographic research, despite its considerable size and the financial contributions it provides to the UK economy. This was certainly the case during and before the 1960s,

therefore the BEVA paper used in this comparison stood alone as the only resource available with which to define the equine population at that time. Currently, there are legal safeguards of animal welfare which did not exist previously (Animal Welfare Act 2006). Additionally, ongoing data gathering operations (e.g. NED, AHT Quarterly Disease Reports) now aim to improve and sustain knowledge of the equine population and its infectious disease status. However, these resources collect no information on the non-infectious disease burden of the population, or on the husbandry and training methods used, and thus the true state of equine welfare in the UK is still not known. This study has provided some direction for further research to pinpoint those areas of equine disease which may be becoming more prevalent, thereby offering priority areas to target for improving the welfare of this population in the future.

Project 3

Cryptosporidium spp., *Giardia* spp., *Salmonellae, Campylobacter* and *E. coli* are examples of enteropathogens able to cause debilitating disease in children, particularly where immunocompromise may occur as a result of concurrent infections such as HIV. For the first time, the GEMS-ZED study is attempting to use case-control methods to uncover associations between domestic animal presence and childhood diarrhoea in rural western Kenya. The conclusions drawn from this study, once complete, will be relevant in many parts of sub-Saharan Africa, and indeed will also be of use in other areas of the developing world. This project furthered this cause by processing a proportion of the animal samples collected, and beginning the analysis of the available data.

The projects undertaken have made modest contributions to the existing bank of information in each of the three fields featured, thus have contributed to the advancement of human and animal health.

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