A cross-sectional survey to investigate prevalence of and clinical indicators for Subacute Ruminal Acidosis (SARA) in lactating cows on UK dairy farms

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Abstract

Subacute ruminal acidosis (SARA) is a commonly described syndrome in dairy cows, with various ill health effects and production losses attributed to it. There has been no previous published study of prevalence in UK dairy cows, and diagnosing the problem is a challenge. Often, clinical indicators such as low body condition scores, excess long fibres in faeces, diarrhoeic faeces and milk fat depression are used as proxy diagnostic indicators for the condition.

This cross-sectional survey collated data collected over several years by a single practitioner during the course of routine clinical practice to determine the prevalence of SARA, as defined by rumen fluid pH ≤5.5 (collected by rumenocentesis). Alternative clinical indicators for SARA were investigated.

The results show that of 244 dairy cows on 22 separate farms between 3 and 271 DIM, 26.2% had rumen pH ≤5.5, but with a large inter herd and inter visit variation (range 0-83%). There was no significant correlation between rumen pH and BCS, DIM, parity, milk fat, yield, sub-clinical ketosis and faecal characteristics.

Logistic regression analysis revealed that two variables, protozoa score (numbers and activity of protozoa in rumen fluid, observed microscopically) and rumen fill score, were significantly associated with the risk of rumen pH ≤5.5. Cows with higher rumen fill scores (>2.5) had a higher risk of low rumen pH (odds ratio 2.65, p-value <0.05). Rumen fluid with higher protozoa scores (more dense population and more motility) had lower risk of low rumen pH, per unit increase in score (odds ratio 0.21, p-value <0.05).

Results suggest that rumenocentesis is a useful and safe procedure to investigate SARA, and using microscopical examination of rumen liquor may be a useful adjunct to measuring pH.

List of Abbreviations

ANOVA	Analysis of variance
BCS	Body condition score
BF	Butterfat
BHB	Beta-hydroxybutyrate
DIM	Days in milk
DMI	Dry matter intake
FC	Faecal consistency
FD	Faecal (fibre) digestion
Hp	Plasma haptoglobin
IQR	Interquartile range
LAL	Limulus amoebocyte lysate
LPS	Lipopolysaccharide
NDF	Neutral detergent fibre
NEFA	Non-esterified fatty acids
NPV	Negative predictive value
P	Probability value
PMR	Partial mixed ration
PPV	Positive predictive value
PS	Protozoa score
Q1	Lower quartile
Q3	Upper quartile
RF(S)	Rumen fill (score)
SAA	Serum amyloid A
SARA	Subacute ruminal acidosis
SCK	Subclinical ketosis
TMR	Total mixed ration
VFA	Volatile fatty acid
X^2	Pearson chi-square test value

Contents

Introduction

Subacute ruminal acidosis (SARA) is a commonly recognised digestive disorder of dairy cows, particularly affecting early to mid lactation cows in high production herds. Kleen et al (2003), Krause and Oetzel (2006), Enemark (2008), and Plaizier et al (2008) have all recently reviewed the subject. The economic effects of SARA are thought to be significant due to decreased dry matter intakes (DMI), decreased yields, and increased prevalence of associated diseases and disorders (Stone, 2004).

Subacute ruminal acidosis is characterised by pH levels that are considered below physiologically normal but which is not the more severe (acute) form of acidosis such as might be caused by accidental cereal over-eating, where the pH is likely to be below 5 (Blood and Radostits, 1994, Owens et al, 1998). It is considered to be very widespread within dairy cow populations (Kleen, 2003, Enemark, 2008), particularly (but not exclusively) in high producing cows fed a cereal-rich diet, such as is common in housed herds. The definition of SARA varies, and comparisons between studies can be difficult. Furthermore, making a diagnosis of SARA, either in an individual animal, or in a herd, is not simple (Bramley et al, 2008; O'Grady et al, 2008; Tajik and Nazifi, 2011).

Rumen pH regulation

In cattle with fully developed fore-stomachs, the normal physiological rumen pH varies between pH 5.5 and 7 (Dirksen, 1979). The principal factor determining the pH is the diet, with cattle fed a cereal based diet (for example feedlot cattle) having a pH typically between 5.5 and 6, whilst forage based diets produce a pH between 6 and 7.2 (Blood and Radostits, 1994). Rumen acidosis in cattle is characterised by abnormally low rumen pH (below 5.5) and principally occurs due to favourable conditions for lactic acid production by Streptococcus bovis, initially, followed by other lactobacilli. Lactic acid is approximately ten times more acidic than the volatile fatty acids (VFA) normally produced in the rumen and results in the normal rumen buffering capacity being overcome (Blood and Radostits, 1994). Factors affecting the buffering capacity include bicarbonate production in saliva, bicarbonate/acid exchange at the rumen papillae, VFA absorption into the portal blood circulation, and other inherent buffering capabilities of the diet and rumen liquor (Dijkstra et al, 1992; Dijkstra et al, 2012). Various

adaptive homeostatic mechanisms exist to avoid acidosis. For example, low ruminal pH can stimulate a greater absorptive surface area to remove VFA more rapidly (Martens et al, 2012)

Low rumen pH can be induced experimentally (Krause and Oetzel, 2005, Dohme et al, 2008 and Plaizier et al 2008) and principally there are two methods: increasing starch, or decreasing physically effective rumen fibre (Khafipour et al, 2009 (a and b), Zebeli, et al, 2008, Zebeli, et al, 2011).

Prevalence of SARA

Many studies of SARA have typically focused on individual rumen-canulated cows, or at least at the individual cow level rather than herd-wide investigations (Geishauser et al, 2012). Some field prevalence data is available from various countries which would indicate the prevalence to be typically between 11% to 28% of cows affected. In the United States, a field study by Garret et al (1997) found up to 19% of early lactation cows and 26% of mid lactation cows were affected by SARA, whilst in a third of herds the prevalence was as high as 40%. In Italy, Morgante et al (2007) investigated prevalence in 12 early lactation cows from each of 10 intensive dairies and found three herds where more than 33% had a rumen pH of ≤5.5. Kleen et al (2009), in a Dutch field study involving 197 cows in 18 herds, found a prevalence of 14%. Cannizzo (2008), in a similar study involving 108 cows from 12 Italian dairy herds, found a prevalence of 18%, whilst O'Grady, et al (2008) found 11% of 144 cows from 12 Irish grazing herds had a rumen fluid pH of ≤5.5. Tajik et al (2009) found a prevalence of 28% amongst 196 cows from 10 herds in northern Iran. All of the previously described studies involved taking a rumenocentesis sample at a single point in time, and used pH ≤5.5 as the threshold value to identify SARA. Geishauser (2012) sampled 432 cows in a single large (1147 cows) German herd using an orally introduced rumen fluid scoop, over three consecutive days, and found no cows with rumen fluid below pH 5.5 (the lowest being 5.9), but the method of sampling probably requires an alternative pH threshold to identify SARA (Duffield et al, 2004). Given that SARA is estimated to be a widespread and economically important condition (Enemark, 2008), prevalence studies, or indeed any herd-level studies, are few in number and small scale. There are no known prevalence studies of SARA in the UK.

Defining SARA and diagnosing SARA using measurement of rumen fluid pH

Plaizier et al, 2008, reviewed the various definitions of SARA: typically a rumen pH ≤5.5 when collected by rumenocentesis, measured approximately 4-10 hours after feeding is considered indicative of SARA (Krause and Oetzel, 2006). Ideally, diagnosis of SARA includes standardisation of time of sampling in relation to feeding or measurement over a prolonged period: Gozho et al, (2007) used a threshold of rumen pH depression of between 5.2-5.6 for at least three hours per day, and this definition is now the preferred one for research purposes (Plaizier et al, 2012). Such a diagnosis can be made in fistulated cows but not in practice. Recent developments in telemetric rumen pH monitoring using rumen or reticular boluses also enables pH to be measured over a period of time (Mottram et al, 2008, Zosel et al, 2010; Sato, Ikeda, et al, 2012 and Sato, Mizuguchi et al, 2012), but the expense of equipment and practicalities of use also currently limits this method to a research setting. To ensure the bolus is placed in the ventral sac of the rumen rather than resting in the reticulum, the bolus should be placed surgically via a rumen fistula (Zosel et al, 2010). Ensuring the electrodes do not become blocked, and ensuring correct calibration of the equipment is also a challenge which needs to be overcome (Gasteiner et al, 2009).

Making a diagnosis of SARA in an individual cow in practice is difficult for several reasons. Firstly, there are different techniques for measuring rumen fluid pH, or obtaining rumen fluid samples (Duffield et al, 2004). Generally, rumen fluid collected by an orally introduced probe will have higher pH (possibly due to saliva contamination, or due to placement of the probe), and will give less consistent results compared with rumenocentesis or direct canulation due to variable sampling sites. Secondly, rumen pH is not constant throughout the day, and varies in relation to feeding times (Krause and Oetzel, 2006, Geishauser et al, 2012), generally being at its lowest between 4 and 10 hours after a main feed or start of a day's first fresh feed availability. Thirdly, the pH of the rumen is not uniform, depending on which part of the rumen is measured (Duffield et al, 2004): lower pH measurements are found in the more ventral parts of the rumen. As pH is effectively a measure of concentration of hydrogen ions, even factors such as recent water intake (resulting in a more dilute rumen fluid) will affect a single time rumen pH measurement.

In addition to these aspects, diagnosis at a herd level is made more difficult by ensuring that enough and a representative cohort of cows are sampled. It has been proposed that to make the diagnosis on a herd level, at least 25% of cows have a pH of 5.5 or below, as measured by rumenocentesis (Garrett et al, 1999; Nordlund et al, 1995). In order to have reasonable confidence of a SARA diagnosis, a herd must have 5 or more of 12 sampled cows with a ruminal pH at or below 5.5, and is considered borderline if two to four cows are at or below 5.5, assuming the herd is sampled at the time of lowest likely pH (6-10 hours after first feed if on TMR diet, or 2-4 hours after a main concentrate feed) (Oetzel, 2003). It is suggested that sampling the most at-risk cows should be done, and these are thought to be freshly calved cows or cows in early lactation (Oetzel, 2003). Conversely, although Geishauser et al (2012), in a single large herd study found that pH was affected by days in milk (DIM), the lowest value was at 77 DIM, thereafter rising to 330 DIM, and was accounted for by higher yields and higher DMI in these cows. However, the pH variation was small (<0.15) and with low significance.

Diagnosing SARA using other rumen fluid characteristics

Al-Zahal et al (2011) showed, in an 8-cow study, a correlation between rumen pH and temperature, and proposed that a ruminal bolus measuring temperature might be beneficial in making a diagnosis of SARA. It was suggested that the temperature range 39 to 41℃ corresponded to a ruminal pH range of 5 to 5.6, indicative of SARA. However, feed and water intakes interfere with the diagnosis (Gasteiner et al, 2009; Al-Zahal et al, 2011). Voluntary feed intakes were reduced when the pH fell, and so the temperature rise was very short-lived (Al-Zahal, et al, 2011).

Rumen lipopolysaccharide (LPS) concentration can increase with low ruminal pH due to lysis of gram negative bacteria (Ghozo et al, 2007; Plaizier et al, 2012). LPS in rumen liquor can be determined using a chromogenic Limulus amoebocyte lysate (LAL) assay (Ghozo et al, 2007; Khafipour et al, 2009a, Zebeli et al, 2012), but is not readily available outside a research setting, and in any case still requires sampling of rumen fluid. The relationship between rumen pH and rumen LPS is not a simple one and the underlying cause of the acidosis (excess concentrate or inadequate fibre) might affect this: high concentrate is more likely to cause increased rumen LPS (Zebeli et al, 2012). Measuring LPS concentration in faeces has been investigated with respect to its relationship to rumen pH, and is reviewed by Plaizier et al, 2012. This method would avoid collection of rumen fluid. Again, the

relationship is not simple, but raised LPS in faeces is more likely with high concentrate feeding than low fibre inclusion (Li et al, 2012).

Kleen et al (2009) examined rumen fluid visually, collected from 197 Dutch dairy cows by rumenocentesis, and scored it qualitatively 1 to 5 based on a composite assessment of colour, smell, consistency, protozoa density (by eye), and protozoa activity (visual assessment of gas bubbles). With the exception of consistency, the rumen fluid scores were correlated with ruminal pH ($P<0.001$) with lower scores occurring more frequently in animals with a pH ≤ 5.5. Higher scores, which would indicate a more biologically active ruminal fluid, occurred more often in the animals with a rumen pH ≥ 5.7.

Low rumen pH is generally associated with reduced methanogenesis (Hook et al, 2011a; Poulsen et al, 2012) and ongoing research into methane detection in dairy cows might contribute to detection of SARA in the future (Lassen et al, 2012).

Diagnosing SARA: blood parameters

If rumen pH affects rumen and intestinal LPS concentrations, and on the basis that the LPS may be absorbed into the portal circulation, it raises the possibility of using blood inflammatory indicators, such as serum amyloid A (SAA) or plasma haptoglobin (Hp), as a diagnostic tool for SARA. A recent meta-analysis by Zebeli et al (2012) of 10 different studies involving small numbers of cows with experimentally induced SARA found that 15-21% of overall variation in SAA concentration could be attributed to rumen pH, whereas the dietary concentrate level accounted for 46% of this variation. In a field study involving 108 cows in 12 commercial Italian dairies, Cannizo et al (2012) found cows with SARA did not have higher Hp or SAA concentrations than the cows with normal rumen pH, and suggested that hepatic detoxification of LPS in the portal circulation protects the cows from a systemic acute phase protein response even when low rumen pH might be associated with a greater risk of LPS absorption. In any case, as Hp and SAA concentrations can be raised due to many factors or stressors, they would not appear to be useful indicators for SARA due to a low specificity (Tajik and Nazifi, 2011).

Further blood parameters were investigated by Brown et al (2000) in 20 steers with experimentally induced acute acidosis and sub-acute acidosis. Data modeling suggested that plasma NEFA concentrations, serum potassium and cholesterol concentrations and serum amylase activity could be useful to distinguish between those animals with no acidosis and those with sub-acute acidosis, but any differences found were not significant. Ceroni et al (2012) found in a small Albanian study that white blood cell parameters altered with SARA (raised WBC), yet this was not a finding by Brown at al (2000), and would in any case would have a low specificity for SARA.

Kleen et al (2003) believed that blood gas analysis could be a useful tool in SARA diagnosis, and certainly the study of 20 steers by Brown et al (2000) showed metabolic acidosis (base excess) and decreased blood pH with the experimentally induced acute acidotic animals. More recently, Gianesella et al (2010a), in a field study involving 216 cows in 20 Italian dairy herds, showed a significant relationship between blood gasses and those cows with low rumen pH (measured by rumenocentesis). Partial pressure of blood CO2 (P CO2) was higher for cows with rumen pH below 5.5, and P O2 was lower. Blood gas analysis requires immediate access to relatively specialised and expensive testing equipment which currently limits its use in the field.

A study of 139 Dutch dairy cows (Kleen et al, 2009) found that cows with SARA (rumenocentesis sample pH ≤5.5) lost more body condition over the calving period than those without. The suggestion was made that those suffering from SARA would be more likely to suffer greater negative energy balance due to reduced rumen performance. Further work suggested by this study was to examine a possible relationship between ketosis (serum Beta hydroxy-butyrate concentration) and SARA. There have been no reports found in recent literature which have examined this relationship.

Diagnosing SARA: urine analysis

Enemark (2008) reviewed the relationship between urine pH and rumen pH, and previous studies indicated a positive correlation. Gianesella et al (2010a) found a significant ($P<0.05$) linear reduction in urine pH with cows grouped into normal pH (>5.8, Group A), sub-optimal pH (5.8-5.5, Group B) and low pH (<5.5, Group C). However the reduction was small: group A pH 8.33+/-0.07; Group C pH 8.14+/-0.25.

Urine pH analysis is non-invasive and easy to do, but will have low specificity to rumen pH as many conditions can lead to aciduria including ingestion of anionic salts.

Diagnosing SARA: milk parameters

The fat percentage of milk is influenced by several factors, including lactation stage, breed and composition of feed (Grummer, 1991). Lowered milk fat content is often used as an indicator of SARA (Enemark, 2008). In fact, the relationship is equivocal. Allen (1997) found a significant relationship between mean rumen pH in experimental, rumen cannulated dairy cows and milk fat percentage when he performed a meta-analysis of several experiments. He expressed the relationship as Rumen pH = 4.44 + 0.46 x milk fat %, and this is oft quoted in subsequent literature. However, none of the cows in the studies had a rumen pH below 5.5. The measurements (n=90) were taken from 23 separate small-scale studies over 40 years, and no detail is given to the criteria for inclusion in the analysis. A recent study involving two rumen canulated cows by Enjalbert et al (2008) demonstrated a reversible and significant milk fat percentage reduction and milk fat yield reduction following induced acidosis. Loor et al (2005) proposed a mechanism for milk fat depression due to low rumen pH increasing biohydrogenation of C18:2n-6 fatty acid to trans-10, cis-12 C18:2 fatty acid, which decreases the expression of genes that encode for most enzymes related to milk fat content and synthesis. The study by Enjalbert et al (2008) was able to partially support this theory by demonstrating that low rumen pH altered the biohydrogenation pathways of various fatty acids (and hence fatty acid profiles within the rumen fluid), but trans-10, cis-12 C18:2 was not strictly dependent on rumen pH. They proposed that the relationship could be more strongly correlated to the starch content of the diet (which may coincidentally lead to low rumen pH).

Other studies with experimentally induced SARA have failed to demonstrate a milk fat reduction (Gozho et al, 1997). Few field based studies exist examining either bulk milk fat content or individual milk fat content and SARA. Tajik et al (2009) found no significant differences between SARA affected and non-affected Iranian dairy cows in fat or protein components in milk, but milk records were available for just 36 cows in this 196 cow study.

The work by Enjalbert et al (2008) was recently followed by a slightly larger 12 cow study by Colman et al (2010) to determine if differential milk fatty acid profile could be used as a diagnostic indicator of SARA. They found that the main fatty acids discriminating between the control and acidotic samples were iso C13:0, iso C16:0, and C18:2 cis-9, trans-11 rather than milk fat content per se or C18:1 trans-10, which have been used previously as indicators of acidosis. They concluded that specific milk fatty acids might have the potential to discriminate acidotic cows and could be used diagnostically in the future.

Diagnosing SARA: faecal characteristics

Krajcarski-Hunt et al (2002) demonstrated that experimentally induced SARA in four ruminally fistulated cows significantly reduced in situ fibre digestion of forages in the diet. Presumably, this could lead to more undigested Neutral Detergent Fibre (NDF), for example cellulose from dietary forage, being evacuated in faeces.

Faecal sieving has been proposed as a diagnostic method for SARA, where faecal samples are collected and sieved under running water using a standard sieve. The presence of larger particles of fibre (greater than 2.5cm), undigested grains, fibrin casts or mucus have been suggested as indicative of SARA (Grove-White, 2004; Hall, 1999 and 2002), but there is no experimental evidence to support this, despite its widespread use in the field.

There isn't a standard method for scoring faeces characteristics but Zaaijer and Noordhuizen (2003) describe a novel scoring system for consistency and fibre digestion (undigested faecal fraction) without a sieve. Mgbeahuruike (2007) describes a more detailed study of faecal characteristics of early lactation dairy cows using wet-sieving methods, and the relationship to different forages in the diet, but does not draw a correlation to rumen pH. Atkinson (2009) describes and illustrates a wet-sieving scoring system, based on Mgbeahuruike's previous study.

In the study by Kleen et al (2009) in 196 Dutch dairy cows, there was no significant relationship between rumen pH and faecal characteristics scored on a 5 point scale based on the scoring system of Zaaijer and Noordhuizen (2003).

Diagnosing SARA: clinical assessment of cows

Grove-White (2004) describes that assessing cows for rumen fill, body condition score (BCS) loss in early lactation, body dirt score, lameness prevalence, faecal characteristics, rumination, fertility and production parameters and the overall health and appearance of the cows within a herd can be used in the diagnosis of SARA. In the field, SARA is often diagnosed on the basis of this general type of assessment (personal observation), but there is no recorded illustration of diagnosis of SARA using these methods either experimentally or in field studies.

Kleen et al (2009) found no correlation between BCS and rumen pH in 196 Dutch dairy cows. However, in 139 of these cows, it was possible to determine a BCS change between three weeks before calving and three weeks after calving, and in those animals, using analysis with a general linear model, cows diagnosed with SARA at a single time rumenocentesis sample taken between 1 and 182 days into lactation had a significantly greater loss in BCS (P=0.026).

Clinical effects of SARA

Regarding the adverse health effects of SARA, there are several conditions which have been attributed to the condition: loss of body weight (Kleen et al, 2003), increased lameness (Nocek, 1997, Cook et al, 2004), loose faeces (Hall, 2002), liver abscesses (Nocek, 1997, Oetzel, 2000, Enemark, 2008), increased inflammatory mediators and circulatory endotoxins (Nocek, 1997, Khafipour et al, 2009 (a), Li et al, 2012, Plaizier et al, 2012), decreased reproductive performance ((Enemark, 2008), and increased risk of abomasal displacement (Olson, 1991). In addition, various production losses are also attributed: depression in milk fat percentage (Allen, 1997, Bramley et al, 2008), reduced dry matter intakes (Brown et al, 2000) and reduced yield (Krause and Oetzel, 2005). However, links with many of these health effects and production traits are disputed, or there is very weak evidence to support them.

Aims of this study

A review of the literature highlights many gaps in the understanding of SARA and its effects, the difficulty of its diagnosis in practice, and its prevalence in UK dairy farms . The objectives of this study were:

- to determine the prevalence of SARA in commercial dairy herds in a central region of the UK.
- to determine whether SARA can be predicted using clinical indicators or less invasive techniques than rumen fluid collection, specifically body condition score, rumen fill score, faecal characteristics, blood beta-hydroxybutyrate concentration, or milk fat percentage.
- to investigate the relationship between rumen pH and rumen fluid protozoal numbers and activity, to act as a preliminary study examining the use of cow side rumen fluid microscopy as a diagnostic tool for evaluating rumen micro-biome health.

Materials and Methods

Herds and Husbandry

Cows from twenty two dairy farms in Shropshire, Staffordshire, Cheshire and North Wales were involved in the study, all being clients of a single veterinary practice, Lambert, Leonard and May, based in North Shropshire. Herds were not specifically recruited onto the study but data were collected during routine or diagnostic "rumen health visits" conducted by the author between January 2006 and July 2012, following a protocol described by Atkinson (2009) during the normal course of clinical veterinary practice. Therefore, the data represent a convenience sample.

Herd size ranged from 120-450 cows (mean 245): see Table 1. Three hundred and five day average yields ranged from 6800 litres to 9700 litres (mean 8466 litres). Average butterfat for all herds was 4.1% and protein 3.25%. All herds were Holstein-Friesian (not necessarily pedigree).

On all herds, cows were fed either a Total Mixed Ration (TMR) or Partially Mixed Ration (PMR) diet consisting of a base forage ration of grass and maize silages mixed with concentrate components in a mixer wagon. Five of the herds were all-year housed fed an exclusively TMR; a further two herds were also fully housed but fed a PMR supplemented either by in-parlour feeding or out-of-parlour feeders. Fifteen herds had a pasture period between May and October, where part of the diet was available from grazed grass. All herds were all-year calving. One herd was loose housed on straw while the remainder were cubicle-housed on deep sand or mattresses.

Table 1: Farms involved in the study

Farm	Herd size	305 day average yields	Management system	Number cows included in study
A	400	8500	housed; TMR	55
B	150	7000	PMR; parlour; SG	7
C	120	8250	PMR; parlour; SG	5
D	300	9200	housed; TMR	24
E	200	7000	PMR; parlour; SG	7
F	300	6800	PMR; parlour; SG	10
G	170	8900	PMR; parlour; SG	10
H	180	8200	PMR; parlour; SG	6
I	170	9700	PMR; parlour; SG	9
J	150	8600	PMR; parlour; SG	10
K	400	9300	housed; TMR	11
L	180	8300	PMR; parlour; SG	5
M	190	8900	PMR; parlour; SG	5
N	200	7800	PMR; parlour; SG	6
O	330	8800	PMR; parlour; SG	6
P	220	8200	housed; PMR; parlour	5
Q	200	8100	housed; PMR; parlour.	8
R	450	9200	housed; TMR	12
S	200	8900	housed; TMR	7
T	170	8800	PMR; parlour; SG	12
U	270	8600	housed; TMR	12
V	450	9200	housed; PMR; OOPF	12
Mean	245	8466		11

Key: Housed = all year housed; TMR = Total Mixed Ration; PMR = Partial Mixed Ration; Parlour = fed concentrate in parlour; SG = Summer Grazing; OOPF = Out Of Parlour Concentrate Feeders

Animals and sampling

Cows were selected by the vet prior to the rumen health visit on the basis of being at high risk of having SARA within the herd, following guidelines suggested by Oetzel (2003): for TMR herds, these would be

cows in days 5-30 of lactation; in PMR herds receiving additional concentrate feeds, cows being fed the maximum concentrate rations on the farm would also be included. Cows were selected from farmers' own records based on calving date and lactation number and a list given to the farmer to present for sampling. The list was limited to the first 12 cows picked in chronological order of calving date (12 cows beginning at 5 DIM for TMR herds; 6 cows beginning at 5 DIM and 6 cows beginning at 30 DIM for PMR herds). When possible, a range of lactation numbers were selected to include some first lactation animals., if necessary substituting up to two multiparous animals for two primiparous cows with greater days in milk.

Sampling visits were timed to coincide with the period of lowest expected rumen pH for the herd. For TMR herds, this was between 4-6 hours after the first fresh feed delivery of the day; where a main concentrate feed was also given, this was 2-4 hours after this feed (typically 2-4 hours after morning milking).

For herds which milk recorded, visits were timed to be within 7 days of a recording date.

All cows were sampled by rumenocentesis (method described in appendix A) to collect 5-10mls of rumen fluid into a sterile plastic screw top container, which was immediately re-sealed. Its pH was measured within 1 hour of collection using a portable pH meter ("pH Checker", Hanna Instruments) whose electrode was allowed to soak and stabilise for 60 minutes in buffered saline before being calibrated immediately prior to use following the manufacturer's instructions, using a buffered pH 4.01 and pH 7.01 solution at room temperature.

Once all rumen fluid samples had been collected, the pots were placed in a warm water bath (37±2℃) for 10-15 minutes before agitating and removing 0.25 ml to examine on a pre-warmed slide with marked coverslip under low power microscopy (x 40) to score the fluid for protozoal density and activity on a 4 point scale (described in appendix B). Protozoal scores were recorded prior to recording pH in order to avoid operator bias.

Cows were scored for BCS on a 1-5 scale using 0.25 increments as described by the DairyCo fact sheet (2011) based on the Penn-State method (DairyCo, 2012).

Cows were scored for rumen fill (RF) on a 1-5 scale using 0.5 increments as described by Atkinson (2009) and Hulsen (2012). (See appendix C)

A faeces sample from each cow was collected into an inverted rectal glove. It was scored for faecal consistency (FC) on a 1-5 scale using 0.5 increments described by Atkinson (2009) and based on a scoring system described by Zaaijer and Noordhuizen (2003). (See appendix D). The collected faeces was scored for "faecal digestibility" (FD) after all cows had been sampled, on a 1-5 scale using 0.5 increments, using a wet-sieve method described by Atkinson (2009) based on a method described by Mgbeahuruike (2007). (See appendix E). Scoring was recorded before measuring rumen fluid pH to avoid operator bias.

A venous blood sample was collected from the cow's coccygeal vein to test for serum BHB concentration, as an indicator of ketosis.

Where individual cow milk constituent recording occurred within 7 days of sampling, milk data from the recording date (butterfat, protein, yields) were later collated for the sampled cows. All milk recordings were carried out independently and commercially by a recording agency certified by the International Committee for Animal Recording. Only non-factored recordings were used.

Twelve cows were selected for each sampling visit, but farmers did not always present all selected cows. Cows were excluded if they were pyrexic or obviously ill. Data were only included for analysis where a rumenocentesis pH result was obtained. A total of 29 sampling visits were included in the data consisting of 244 cow samples on 22 different farms, with a median of 8 cows per visit (range 4-12). One cow (from farm A) was sampled on two occasions, 2 years apart. On a few occasions, later lactation cows were presented in error, but these were also sampled and included in the study. The median days in milk was 28 days (range 3-271).

Test considerations

Rumenocentesis samples that were contaminated by blood were discarded. All pH measurements were taken within 1 hour of sampling. Loss of carbon dioxide from samples into the atmosphere can increase pH and immediate pH measurement is recommended (Duffield et al, 2004) to reduce the chance of falsely raised pH measurements. A previous pilot study showed that a 1 to 2 hour delay in taking pH measurements did not alter the results.

Herds using dietary milk butterfat moderators, such as addition of protected C-16 fat supplements, were included the study.

Blood for BHB analysis was either collected into a labelled serum tube (BD Vacutainer) and sent by overnight post to a commercial laboratory (NUVetNA, School of Veterinary Medicine and Science, University of Nottingham) for serum BHB assay using a clinical chemistry analyser and a commercial assay kit, (Rx IMOLA, Randox Laboratories) or, in the majority of cases, the serum BHB concentration was assessed cow-side using a drop of blood on a commercial test strip (Optium B-Ketone test strip) and a hand-held meter (Optium Xceed ®). The cow-side test relies on first a biochemical reaction of blood BHB, and secondly an electrochemical reaction which releases electrons and generates a current proportional to the serum BHB concentration which is detected by the meter after 10 seconds. The meter is calibrated to show the BHB concentration in mmol/l, and the test has been shown to have a correlation coefficient of 0.97 with commercial laboratory assays (Voyvoda and Erdogan, 2010).

BCS, RF, FC and FD scores are all subjective and liable to operator bias and variability. The same operator (Owen Atkinson) was used to record all scores, and pH was measured last to reduce the chance of bias with respect to knowledge of the pH result.

Protozoal scores are also subjective and liable to error and operator bias. The scoring system has not been described by other operators and its repeatability is uncertain. A small pilot study of 12 samples scored on two separate occasions (2 hours apart) showed 10/12 samples were scored the same, and 2/12 were scored one point above or below their previous score. An additional pilot study showed that the temperature of the fluid prior to scoring affected the protozoal motility greatly. A consistent

temperature of rumen fluid (37±2℃) was used during the study and the same operator was used for all scores (Owen Atkinson). pH was measured after recording scores to reduce the chance of bias.

Ethical and legal considerations

All data were collected during the course of normal clinical practice. Sampled cows belonged to herds where SARA was suspected, and a diagnosis was being sought, or where SARA was being monitored as part of a nutrition advice service. Farmers were given a written report following each sampling visit (called a "rumen health visit"), which included specific management and dietary advice where appropriate. Nutritional advisers to the herds were sent a copy of reports, or actively consulted.

Rumenocentesis and blood sampling are invasive procedures. Local anaesthesia and suitable restraint was used prior to rumenocentesis. Where samples were contaminated with blood or the procedure caused signs of agitation in the cows, rumenocentesis was not re-attempted.

Data handling and statistical analysis

Data were initially collected onto hard copy data-collection sheets. These were later collated, along with milk recording data, in a spreadsheet (Microsoft Excel 2007; Microsoft Corp.). Data were only included if a rumen pH result was available. One hundred and forty four cows had a complete data set of rumen pH, milk yield and constituents, FC score, FD score, RF score, BCS, BHB concentration and protozoal score.

A commercial statistical software package (Minitab 16; Minitab Inc.) was used for initial statistical analysis. The distribution of rumen pH values was described, and simple linear regression analysis used to compare pH in cows with different yields, days in milk (DIM), milk butterfat percentage and BHB concentrations. A straight line of best fit was used, calculated with the lowest squared residuals. One-way analysis of variance (ANOVA) was used to compare rumen pH within categories of parity, farm, visit occasion, BCS, RF score, FC score, FD score and protozoal score. In these instances, multiple box plots were used to represent the data graphically. Each box plot shows the range of pH values within a category (top and bottom whiskers), the median (horizontal line in the box) and the lower (Q1) and

upper (Q3) quartiles (margins of the box). Single outlying results are represented by an asterisk, and in these cases the lower whisker limit are values falling within Q1-1.5xIQR, and the upper whisker limit are values within Q3+1.5xIQR.

Cows were grouped into binary categories (0/1) for SARA based on a rumen pH ≤5.5 which was used as the "gold standard" SARA diagnosis. Other binary categories created were cows which were diarrhoeic (FC score ≤2); had poor fibre digestion (FD score ≥3); had subclinical ketosis (BHB ≥1.2mmol/L); were thin (BCS ≤2); had empty rumens (RFS ≤2); had low butterfat percentage (two thresholds: BF ≤2.5% and BF ≤3.49%) and cows with poor protozoal scores (protozoal score ≤1). (See appendix F for explanation of cut-off points). Pearson Chi-square tests (X^2) were used to evaluate the relationship between commonly used clinical indicators for SARA and the gold standard: low BCS, empty rumens, diarrhoea, low butterfats, and poor fibre digestion. Two by two contingency tables were constructed for each parameter and positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity calculated to ascertain the degree of association of each clinical binary parameter with the gold standard SARA diagnostic test. (See appendix G for explanation of the Pearson Chi-square test, p-value, PPV, NPV, sensitivity and specificity in a 2 x 2 contingency table).

An alternative test for SARA diagnosis, individual cows with protozoal score ≤1, was also tested against the same clinical indicators using Pearson's Chi-square test.

As a final step, multivariable statistical analysis was used. A conventional multilevel logistic regression model was constructed to evaluate factors that influenced the risk of a rumen pH ≤5.5. This analysis accounted for the correlation between cows within farm and therefore adjusted for the lack of independence between cows. The model was constructed using MLwiN (version 2.26, University of Bristol). Explanatory variables were carried forward into the model only where initial Chi-squared analysis revealed a p-value <0.2. Variables remained in the final logistic regression model when p-value was ≤0.05. The relationship between explanatory variables and low rumen pH (≤5.5) was expressed as an odds ratio.

A significance probability, P ≤0.05 was used to indicate significance (α).

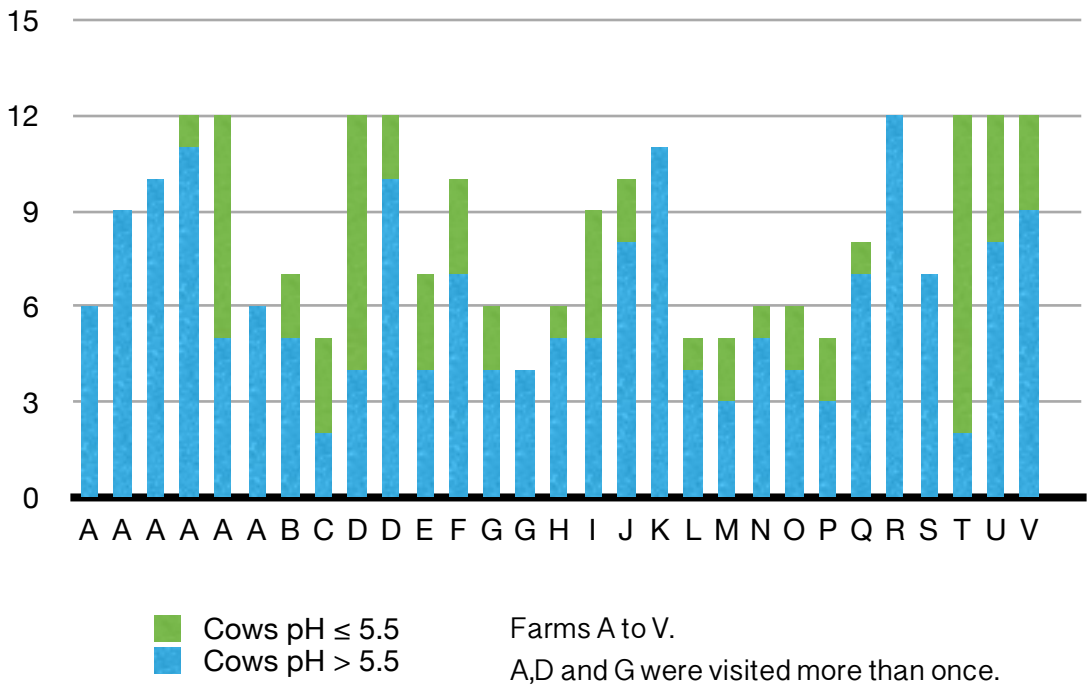
Results

A total of 244 cow rumen pH results were obtained from 22 different farms over 29 sampling visits between 2006 and 2012.

SARA prevalence

Sixty four rumenocentesis samples were ≤5.5 pH, indicating that 26.2% of sampled cows were affected by SARA at the time of the visit. At least one cow with SARA was found on 21 of the 29 visits: see Figure 1. At only 8 visits were 12 cows sampled, allowing a herd diagnosis to be reached by the criteria described by Oetzel (2003) whereby at least 5/12 cows must be ≤pH 5.5 to indicate a likely herd prevalence of 25%: this occurred on 3 occasions on farms A, D and T (see Table 2).

Figure 1: Chart of all sampling visits showing total number sampled and proportion above and below pH 5.5



The within visit prevalence of SARA ranged from 0 to 83% of sampled cows.

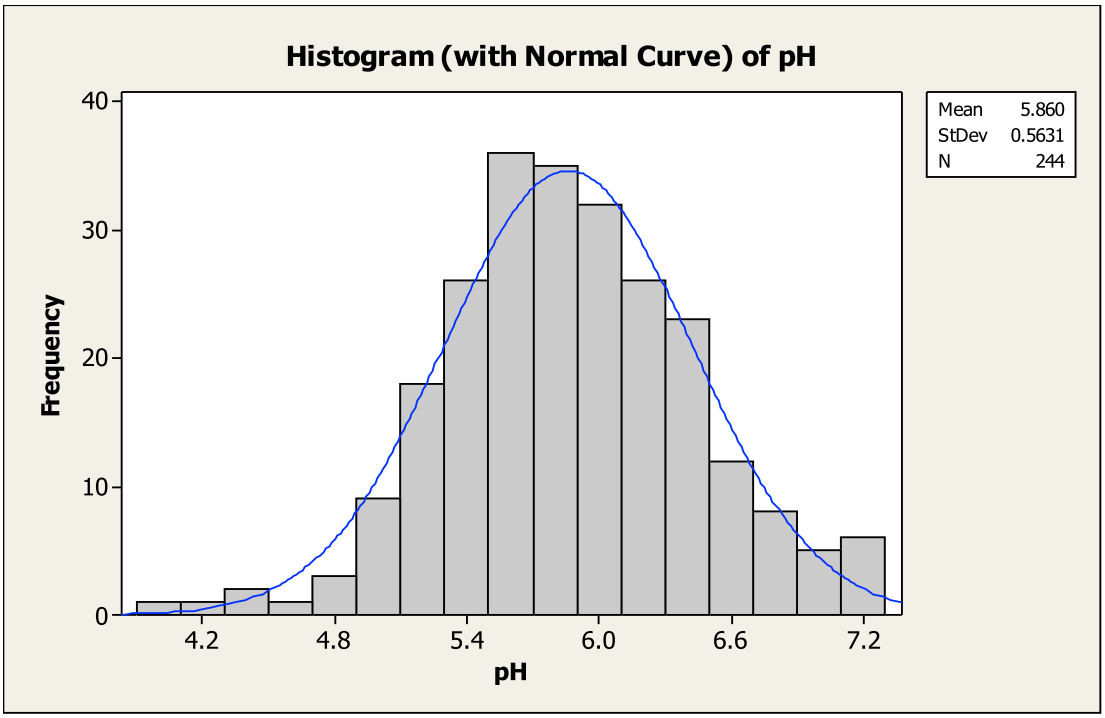
Table 2: Rumen fluid pH value ranges for each sample visit, and prevalence of SARA

Farm visit	Number of cows within each ruminal pH range					Number of cows sampled and SARA prevalence	
	pH ≤ 5.5	5.51-5.8	5.81-6.1	6.11-6.4	≥ 6.41	n	prevalence SARA
A1	0	3	0	1	2	6	0
A2	0	1	1	4	3	9	0
A3	0	1	1	3	5	10	0
A4	1	3	1	3	4	12	0.08
A5	7	3	1	0	1	12	0.58 *
A6	0	0	2	3	1	6	0
B	2	0	1	3	1	7	0.29
C	3	1	1	0	0	5	0.60
D1	8	4	0	0	0	12	0.67 *
D2	2	3	4	1	2	12	0.17
E	3	2	1	0	1	7	0.43
F	3	3	2	2	0	10	0.30
G1	2	3	0	1	0	6	0.33
G2	0	3	0	1	0	4	0
H	1	1	3	1	0	6	0.17
I	4	1	0	2	2	9	0.44
J	2	4	3	1	0	10	0.20
K	0	0	2	0	9	11	0
L	1	2	2	0	0	5	0.20
M	2	3	0	0	0	5	0.40
N	1	1	0	2	2	6	0.17
O	2	3	1	0	0	6	0.33
P	2	1	1	1	0	5	0.40
Q	1	1	5	1	0	8	0.13
R	0	1	6	3	2	12	0
S	0	1	1	4	1	7	0
T	10	1	0	0	1	12	0.83 *
U	4	4	3	1	0	12	0.33
V	3	3	1	2	3	12	0.25
Sum	64	57	43	40	40	244	26.2%

* indicates herd level SARA likely to be >25%

The range of rumen pH measurements was 4.02 to 7.28. Mean was 5.86 and median 5.83. The pH values were approximately normally distributed around the mean (standard deviation 0.56) (Figure 2): 95% of data lie in the range of mean ± 1.96 x standard deviation.

Figure 2: Chart showing the distribution of rumen pH measurements in 244 cows

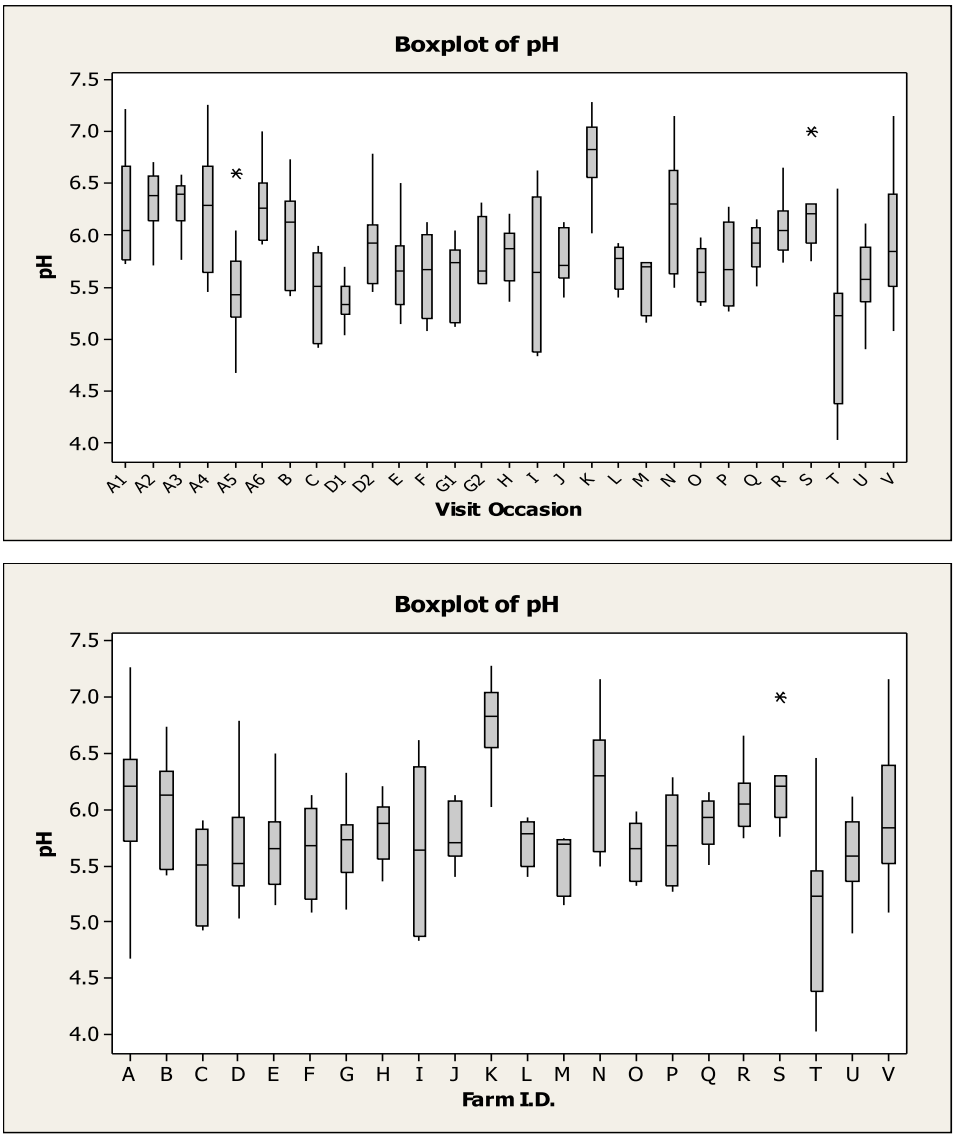


Lower quartile threshold (Q1) = 5.49 Upper quartile threshold (Q3) = 6.24

Farm effects and visit effects

The farm and visit occasion did affect ruminal pH. For visit occasion, a one-way analysis of variance (ANOVA) showed F to be 6.55 with a P-value of <0.01. For farm, F was 5.84, with a P-value of <0.01. Therefore, both farm and visit showed significant variation in pH values, with visit occasion having a slightly greater effect. Figure 3 shows the median and distribution of pH values at each visit occasion and for each farm, in multiple modified box plots.

Figure 3: Box plots showing the distribution of rumen pH measurements for each visit occasion and each farm.



One-way ANOVA showed that for the three farms which had more than one visit occasion (A, D and G), there was a significant effect of the visit occasion on the rumen pH for two of the farms (A and D). Farm G had very small sample sizes on both occasions.

Farm A: F = 5.84 P-value < 0.001

Farm D: F = 17.57 P-value < 0.001

Farm G: F = 0.63 P-value 0.451

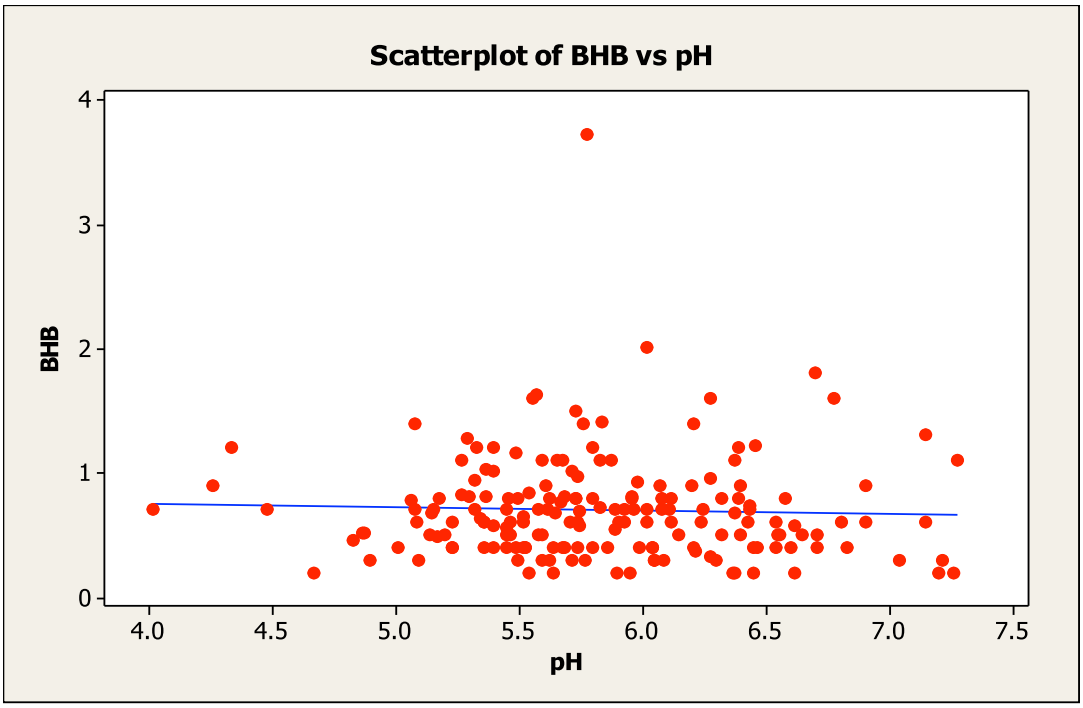
Regression analyses for pH and BHB, DIM, yield, and BF.

Beta-hydoxybutyrate:

One hundred and seventy nine cows had a BHB concentration result. Twenty (11.2%) had subclinical ketosis (SCK), using the threshold criteria of blood BHB concentration ≥ 1.2mmol/l as indicative of SCK (Duffield et al 2009).

Regression analysis showed no statistical correlation between BHB concentration and pH (see Figure 4).

Figure 4: Scatter plot of BHB and pH values

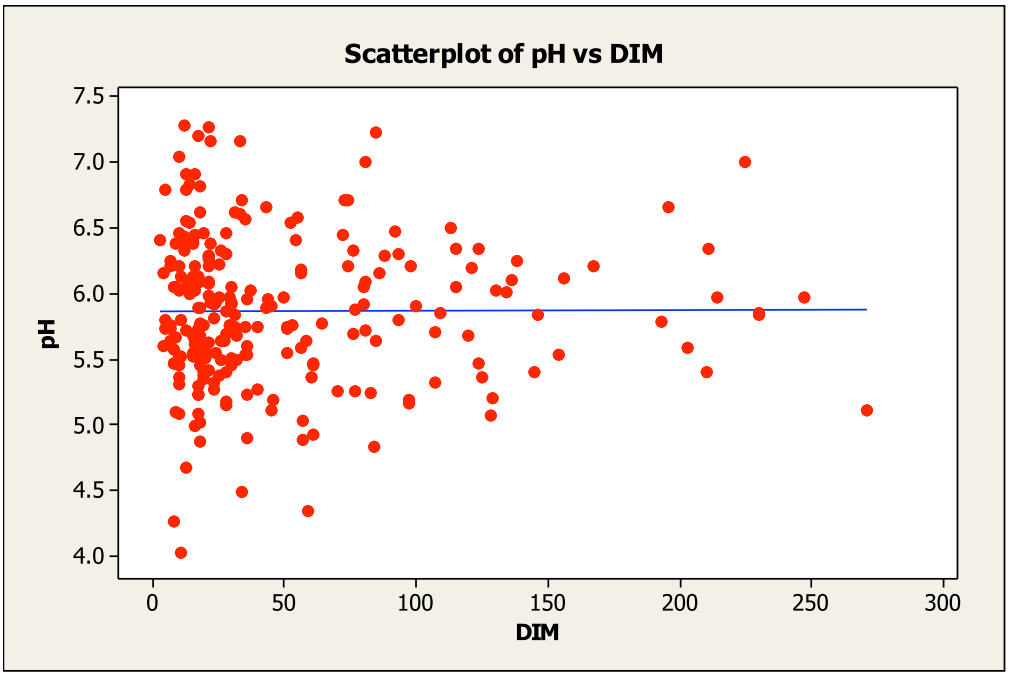


R^2 0.1% P-value 0.64 (Blue line is the line of "best fit" based on squared residuals)

Days in milk:

The majority of samples were taken during early lactation (median 28, range 3-271). The data did not show a correlation between DIM and pH (Figure 5).

Figure 5: Scatter plot of pH and DIM

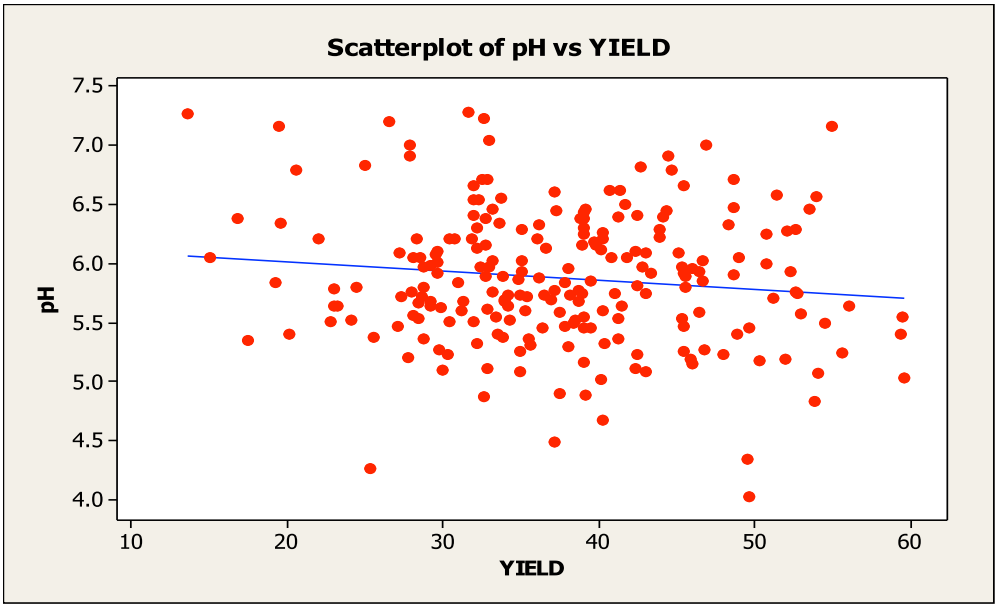


R^2 0.0% P-value 0.922

Milk yield:

A milk yield was available for 224 cows. The mean yield was 37.6 litres, median 37.4 litres, and range 13.6-59.6 litres. There was a weak but non-significant correlation between yield and rumen pH, with a tendency for higher yields to be associated with lower pH (Figure 6).

Figure 6: Scatter plot of pH vs yield (litres per day)

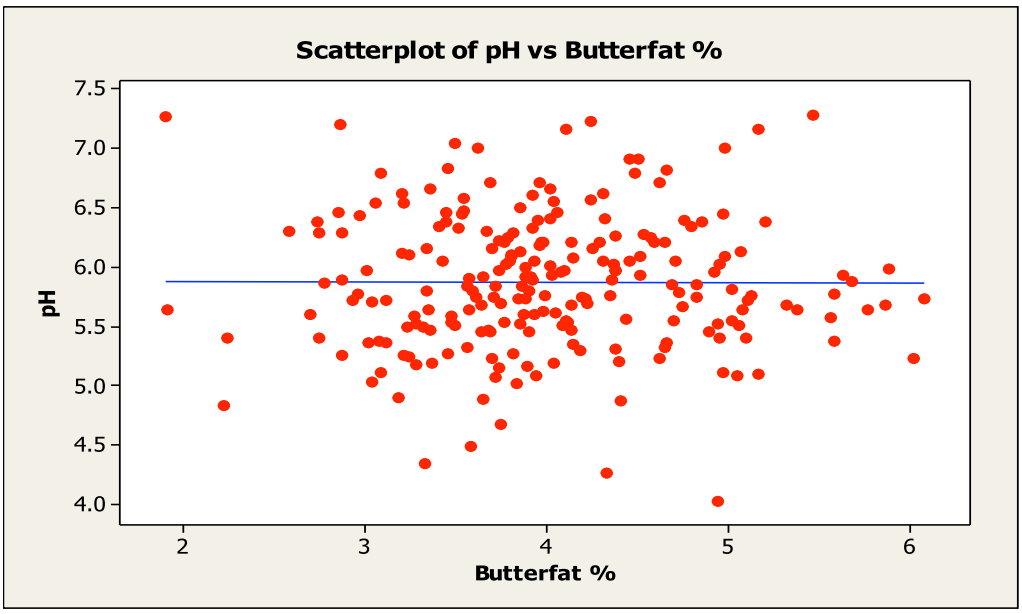


R^2 1.6% P-value 0.060

Milk butterfat:

Milk constituent values were available for 224 cows. The range of butterfat percentage was 1.9% to 6.08%, mean 4.01%, median 3.92%. There was no significant correlation between butterfat% and rumen pH (see Figure 7), although mean BF for SARA cows was 3.89%, against a mean of 4.05% for non-SARA cows.

Figure 7: Scatter plot of pH vs milk butterfat %



R^2 0.0% P-value 0.924

The simple linear regression line of best fit is horizontal, indicating no tendency for higher or lower butterfats being correlated with lower pH values.

Associations of rumen pH with categories of parity, BCS, RF, FC and FD scores.

Parity:

The lactation number was known for 232 of the cows. There was a weak correlation between SARA and parity number, with 3rd lactation cows having the greatest prevalence but this was not significant (Pearson Chi-square = 3.073, P-value 0.381): see Table 3.

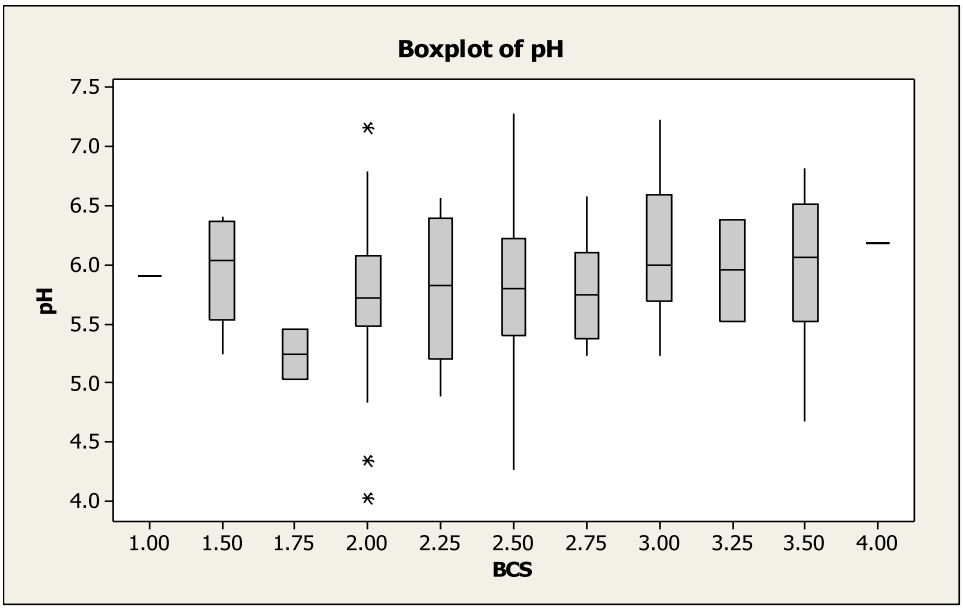
Table 3: Cows by lactation number and prevalence of SARA

Lactation Number	Number sampled	Number pH ≤ 5.5 (SARA)	Percentage SARA
1	38	8	21.1
2	80	22	27.5
3	47	16	34.0
≥4	67	14	20.9

Body condition score:

A BCS was available for 225 cows. The range was 1 to 4 (mean 2.48; median 2.5). There was no association found between BCS and pH (Figure 8).

Figure 8: Boxplot of pH for cows in different BCS categories



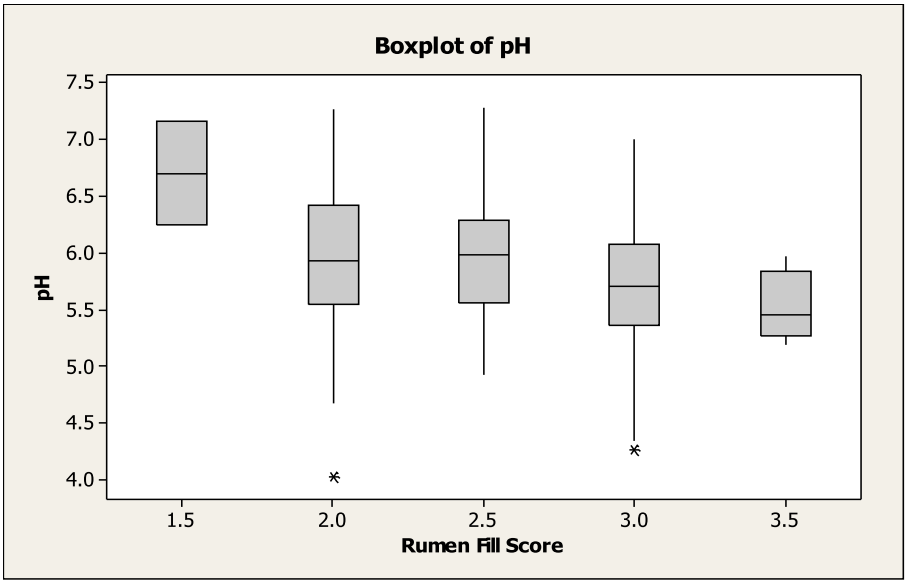
One-way ANOVA F = 1.13 P-value 0.339

There were only single cows with BCS score 1 and 4. The range of pH values for each BCS category is shown by the vertical whiskers and asterisks. The upper and lower quartile thresholds are shown by the extents of the boxes, and the horizontal lines are the median. An asterisk represents an outlying result.

Rumen fill:

Two hundred and twenty nine cows had a rumen fill (RF) score. One-way ANOVA showed a significant effect ($P<0.01$) of RF score category on rumen pH. Cows with higher RF scores tended to have lower pH values. Figure 9 shows the range and medians of pH values within each RF score category: cows with SARA occurred within all categories except RF score 1.5 (n=2), but more so with higher scores.

Figure 9: Boxplot of pH for cows in different Rumen Fill score categories



One-way ANOVA F = 5.06 P-value 0.001 R^2 = 8.29%

The distribution of cows within the RFS categories was not even, as shown in Table 4:

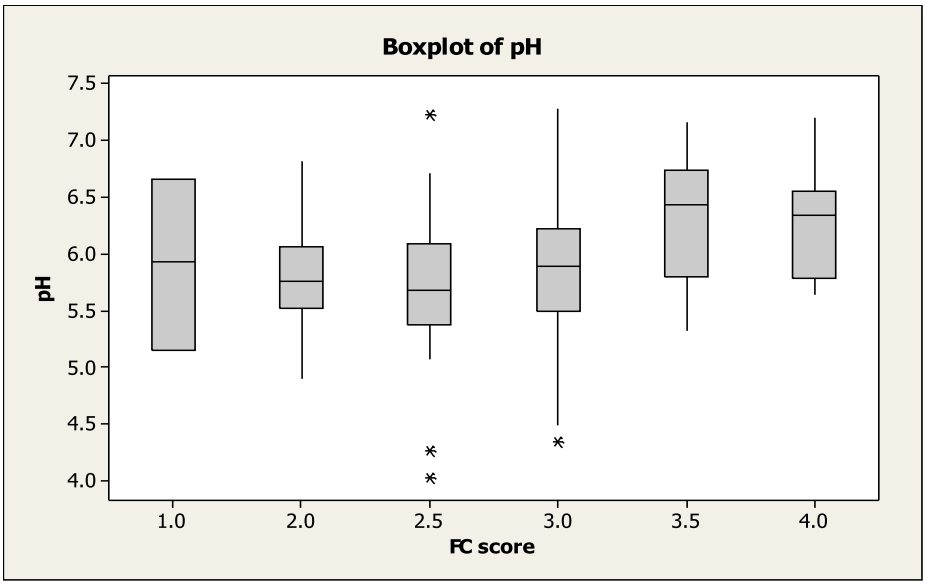
Table 4: Cows by Rumen Fill Score

Rumen Fill Score	Number, n	Mean pH	Standard deviation
1.5	2	6.69	0.64
2.0	58	5.96	0.62
2.5	69	5.98	0.55
3.0	92	5.70	0.53
3.5	8	5.52	0.30

Faecal characteristics:

Faeces was scored for consistency (FC) and "fibre digestion" (FD). Two hundred and thirty six cows had scores for both FC and FD. There was no correlation between FC and FD (one-way ANOVA F = 0.51, P-value 0.80). There was no statistically significant correlation between pH and FD score (Figure 11), but a significant relationship (P<0.05) existed between pH and FC score category, with an FC score of 2.5 tending to have the lower pH values (Figure 10).

Figure 10: Boxplot of pH for cows in different FC score categories



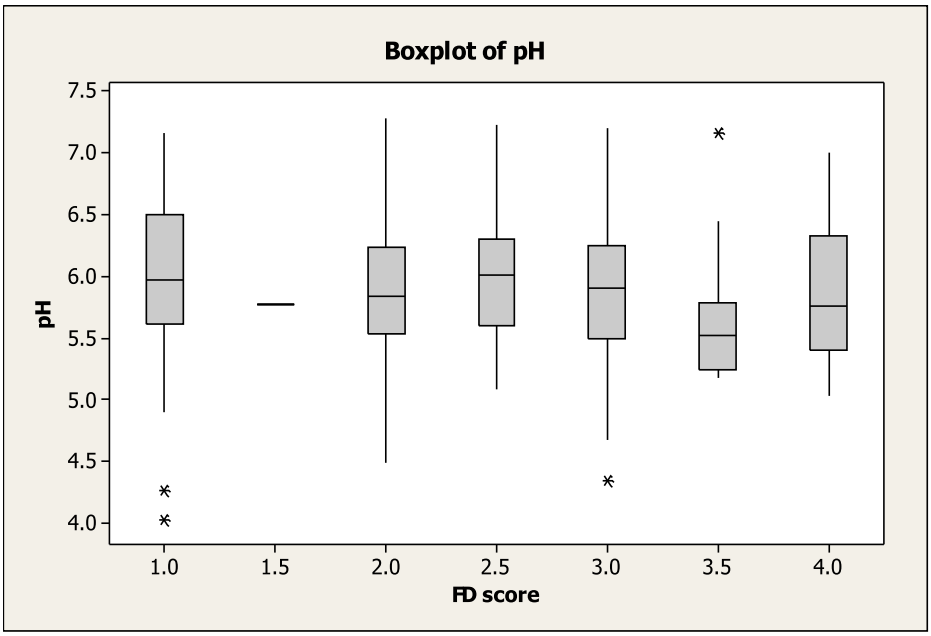
One-way ANOVA F = 2.56 P-value 0.028

The distribution of cows within FC score categories was uneven (Table 5):

Table 5: Cows by Faecal Consistency Score

Faecal Consistency Score	Number, n	Mean pH	Standard deviation
1.0	3	5.91	0.76
2.0	37	5.83	0.48
2.5	31	5.66	0.63
3.0	153	5.86	0.55
3.5	8	6.30	0.59
4.0	7	6.26	0.55

Figure 11: Boxplot of pH for cows in different FD score categories



One-way ANOVA F = 0.82 P-value 0.552

Protozoa score and rumen pH

One hundred and ninety eight cows had a protozoa score for their rumen liquor. Table 6 shows the distribution of individual cows in each protozoal score category, where score 0 represents no or negligible protozoa detectable in the rumen liquor and score 3 represents large numbers of big and small protozoa with good (vigorous) motility. The percentage of SARA cows in each category is also shown.

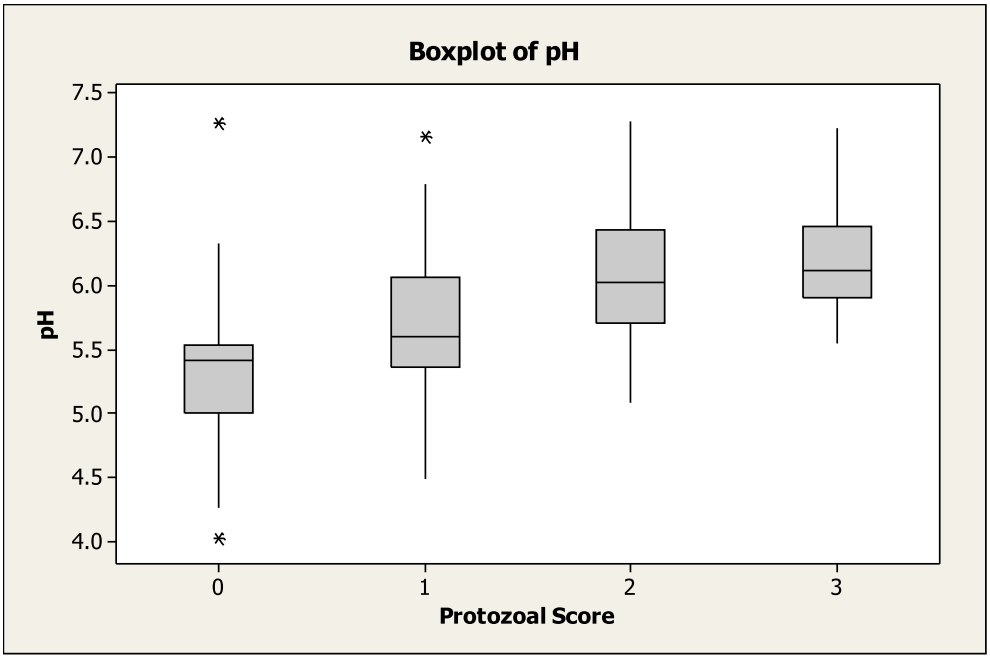
Table 6: Cows by protozoa score and prevalence of SARA

Protozoa score	Number in category	Number pH ≤ 5.5 (SARA)	Percentage SARA
0	36 (18.0%)	25	69.4
1	49 (24.7%)	21	42.9
2	67 (33.8%)	8	11.9
3	46 (23.2%)	0	0

The Pearson Chi-square for these tabulated statistics showed a significant relationship between protozoa score and SARA (Pearson Chi-square = 63.47, P-value < 0.001).

One-way ANOVA of the full range of pH values and protozoal score also demonstrated a significant relationship (Figure 12), with lower protozoal scores being correlated significantly with lower pH values ($P<0.01$).

Figure 12: Boxplot of pH for cows in different protozoa score categories



One-way ANOVA F = 26.73 P-value < 0.001 R^2 = 29.24%

A 2 x 2 contingency table was used to compare agreement of two tests for SARA: rumen pH ≤5.5 and protozoa score (PS) 0 and 1 (a binary division: PS 0 and 1 indicating poor protozoa (1), PS 2 and 3 indicating good protozoa (0)). An alternative binary division of PS was also tested whereby protozoal score 0 was taken to indicate SARA (1), and scores 1, 2 and 3 = 0 (no SARA). Comparison of both thresholds against the Gold Standard SARA test are shown in Table 7. The correlation between both tests was significant (P<0.01). Reducing the threshold for protozoal score (PS = 0) increased the specificity of the test to 92.4% from 72.9%, but reduced the sensitivity from 85.2% to 46.3%.

Table 7: Results of comparison of Protozoal Score tests using a 2 x 2 contingency table to calculate Pearson Chi-square correlation (X^2), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), against pH ≤5.5 as the Gold Standard.

Alternative SARA tests: Protozoal Scores	Prevalence % (n)	Gold standard test for SARA					
		rumen pH ≤ 5.5					
		X^2	p-value	Sensitivity	Specificity	PPV	NPV
Protozoal score ≤ 1	42.9 (198)	54.113	<0.001*	85.2%	72.9%	54.1%	92.9%
Protozoal score = 0	18.2 (198)	39.452	<0.001*	46.3%	92.4%	69.4%	82.1%

* indicates significance (p-value < 0.05)

Using the threshold PS ≤1 as indicative of SARA, compared to rumen pH ≤5.5, the overall accuracy of using protozoal score to test for SARA was 76.3% (151 occasions in agreement out of 198 events). A Kappa value was also calculated which accounts for expected random agreements: this was 0.49, indicating a moderate agreement of tests (see appendix G).

SARA and possible clinical indicators: diagnostic agreements

Using rumen fluid pH ≤5.5 as the gold standard diagnostic test for SARA, possible clinical indicators were assessed by 2 x 2 contingency tables for their diagnostic potential. The same indicators were also tested against an alternative test for SARA diagnosis of protozoal scores ≤1 (Tables 8 and 9).

Table 8: Pearson Chi-square (X^2) and p-values for different clinical indicators against two alternative tests for SARA. The prevalence of each indicator is indicated, in the population, n, for which data was available.

Potential clinical indicator	Prevalence % (n)	Test for SARA			
		rumen pH ≤ 5.5		Protozoal score 0 or 1	
		X^2	p-value	X^2	p-value
Diarrhoea (FC ≤ 2)	16.7 (239)	0.535	0.464	4.186	0.041**
Poor fibre digestion (FD ≥ 3)	53.8 (240)	3.261	0.071*	2.548	0.110
Thin (BCS ≤ 2)	21.3 (225)	1.918	0.166*	6.017	0.014**
Empty rumen (RFS ≤ 2)	26.2 (229)	3.146	0.076*	0.386	0.534
Ketotic (BHB ≥ 1.2)	11.2 (179)	0.179	0.672	3.258	0.071
Butterfat <2.5%†	1.8 (224)	1.294	0.255	1.808	0.179
Butterfat <3.5%	25.4 (224)	3.746	0.053*	2.245	0.134
BF/protein ratio < 1	9.8 (224)	0.043	0.836	2.149	0.143

† only 2 cows in the sample had BF <2.5%, so an alternative threshold of 3.5% was also used. This represented the lower quartile of cows in the dataset (Q1 = 3.49%)

*indicates p-value < 0.2; these variables were carried forwards into a multilevel logistic regression model for rumen pH ≤5.5

**indicates significance (p-value < 0.05)

Table 9: Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for clinical indicators against two alternative tests for SARA.

Potential clinical indicator	Test for SARA							
	rumen pH ≤ 5.5				Protozoal score 0 or 1			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
Diarrhoea (FC ≤ 2)	13.8	82.2	22.5	71.9	21.7	89.1	60.0	60.1
Poor fibre digestion (FD ≥ 3)	63.5	49.7	31.0	79.3	56.6	55.0	48.5	62.9
Thin (BCS ≤ 2)	37.1	72.4	33.8	75.2	39.7	77.2	57.4	62.4
Empty rumen (RFS ≤ 2)	17.7	70.7	18.3	69.8	25.0	70.9	40.0	54.9
Ketotic (BHB ≥ 1.2)	9.6	88.2	25.0	70.4	4.6	86.6	21.4	53.4
Butterfat <2.5%†	3.5	98.8	50.0	75.0	3.8	99.1	75.0	58.6
Butterfat <3.5%	35.1	77.8	35.1	77.8	33.3	76.6	51.0	61.2
BF/protein ratio < 1	10.5	90.4	27.3	74.8	14.1	92.5	57.9	59.6

† only 2 cows in the sample had BF <2.5%, so an alternative threshold of 3.5% was also used. This represented the lower quartile of cows in the dataset (Q1 = 3.49%)

All values are expressed as a percentage.

Table 8 shows that there was no significant correlation between any of the diagnostic indicators and the SARA gold standard diagnostic test of rumen pH ≤5.5. There was a weak but significant correlation between diarrhoeic ($P<0.05$) and thin ($P<0.05$) cows and an alternative test for SARA of poor protozoal scores (PS ≤ 1). However, the results in Table 9 suggest that the predictive values and sensitivities and specificities of the clinical indicators are not strong enough to be of reliable practical use, whichever test for SARA diagnosis is used.

The null hypothesis that SARA cannot be usefully predicted by these clinical indicators is supported.

Multivariable statistical analysis

When protozoal score, butterfat percentage, body condition score, faecal fibre digestion and rumen fill score were taken forwards into a multilevel logistic regression model, only protozoal score and rumen fill score remained as accounting for significant effects on risk of SARA (rumen pH ≤5.5). One hundred and seventy three cows were included in the final model. Table 10 shows the odds ratio and p-value for these two variables on the likelihood of low rumen pH (≤5.5).

Table 10: The odds ratios for the two explanatory variables significantly associated with risk of low rumen pH (≤5.5)

Explanatory variable	Odds ratio	p-value
Protozoal score (per unit increase)	0.21	<0.05
Rumen fill score > 2.5 (compared to ≤ 2.5)	2.65	<0.05

Notes: 1: Intercept value 1.2

2: Farm was included in the model as a random effect to account for correlation of cows within herds

3: A higher odds ratio resulted when using RFS threshold of 2.5 as opposed to 2, which is the cut-off previously used

Discussion

Prevalence of SARA: cow and herd level results

The study provides a useful cross-sectional survey of prevalence of SARA amongst lactating cows in dairy farms in the Cheshire/Shropshire region of UK. The overall cow prevalence of 26.2% is in line with similar prevalence studies (Table 11) .

Table 11: SARA prevalence in cross-sectional surveys using rumenocentesis and a rumen fluid pH ≤5.5 as the diagnostic threshold.

Country	Number of cows	Prevalence	Reference
Netherlands	197	14%	Kleen et al, 2009
Iran	196	28%	Tajik et al, 2009
Italy	108	18%	Cannizzo, 2008
Ireland	144	11%	O'Grady et al, 2008
USA, Wisconcin	150	26%	Garrett et al, 1997
UK	**244**	**26.2%**	**This study**

The herds selected in the study were not random: they were all undergoing a "rumen health investigation" as part of normal clinical practice. In some instances, this was routine monitoring as part of an approach to dietary management of the herds, whilst in others, a SARA problem was either suspected or needing to be ruled out as a differential diagnosis (for herd milk yield reduction, for example). Therefore, there is a likely bias towards herds where SARA exists. Further to this, cows within the herds were selected on the basis of being most likely to have SARA: those in early lactation, or those on maximum dietary concentrate. It is worth noting that cows that one might most suspect as suffering from SARA might not be the ones with the lowest rumen pH, and thus this selection process might be of little significance, particularly with regard to days in milk for example.

This study shows that predicting SARA from days in milk is not reliable. Garrett at al (1997) showed that the prevalence of SARA in mid-lactation cows was greater than early lactation cows, and Geishauser

et al (2012) showed rumen pH within a single herd was not significantly affected by DIM, though was generally lowest at 77 DIM. Krause and Oetzel (2006) also reported a non-significant decrease in pH with DIM (up to 140 days), presumably mirroring increases in DMI.

Twenty one out of 29 sampling visits had at least one cow with SARA. Many of the visits did not include enough cows to reliably predict herd SARA prevalence; of the 8 occasions where 12 cows were sampled, 3 had > 33% cows with pH ≤ 5.5, which would suggest SARA was present in the herd at a prevalence of ≥ 25% (the criteria for diagnosing a herd SARA problem, Oetzel, 2003). Although a small sample size, 37.5% (3 out of 8) of herds with SARA is in line with two previous small scale studies: Garrett et al (1997) found 33% of herds had SARA, and Morgante et al (2007) found 3 of 10 Italian dairy herds fulfilled the criteria. None of the studies included a randomly selected group of herds.

The prevalence of SARA within a herd is likely to change depending on many circumstances, primarily but not necessarily confined to feeding practices and diet composition. Three herds, A, D and G, were sampled on more than one occasion, and the SARA prevalence varied on each occasion. This was significant for herds A and D. In this study, the variability between visits was greater than the variability between farms. The practical implication of this is that many farms might have periods of low rumen pH and so the overall proportion of farms suffering periods of SARA each year (incidence) might be higher than the prevalence of 37.5% indicated in this study.

Rumenocentesis

There were no serious adverse reactions and no cow ill-effects reported after the rumenocentesis procedure throughout the course of the study. Farm G, on the first visit occasion, reported 2 (out of 6) cows with small swellings at the rumenocentesis sites. These were examined the following week and thought to be as a result of the injection of local anaesthetic at the site and were non-painful. Cows tolerated the rumenocentesis very well, but care was always taken to anaesthetise the skin and muscle layers prior to insertion of the needle, and a long (4") 16G single-use needle was always used. The technique requires minimal restraint (it can be done at self-locking yokes, but a crush is better), and moderate skill. The safety and lack of ill effects of rumenocentesis on cows found in this study supports previous similar findings (Nordlund, Cook and Oetzel, 2004; Gianesella et al, 2010b), and

suggests this is a technique which can be readily used in practice. Short needles should be avoided to reduce risk of laceration injury to the rumen wall.

A "gold standard" test for SARA

There is no gold standard test for SARA diagnosis in practice. Whilst a consensus has settled on the use of rumenocentesis timed to occur in relation to feeding at the likely nadir of rumen pH, and the use of a threshold of pH ≤5.5, this is likely to be a flawed method. Rumen pH is not a stable entity and there are many potential influences on pH, as described earlier. In fact, a cow with an abnormally low rumen pH will most likely self-correct very quickly as soon as she stops eating. This study gives further support to previous evidence that low pH is associated with large meal intakes: multivariable analysis showed cows with more full rumens (RFS>2.5) were more likely to be acidotic (pH ≤5.5) compared with less full cows (RFS≤2.5) (odds ratio 2.65, p-value <0.05). This might account for higher yielding cows in this study tending to have lower pH (p-value 0.06, non-significant), presumably as they have larger DMI's. This is consistent with findings of Geishauser et al (2012). Presumably, larger feed intakes result in more VFA's from rumen fermentation, which reduces pH.

If low pH is only short-lived, or self-corrects quickly, rumenocentesis and single-time pH measurement will have a very low sensitivity in detecting cows with SARA. Indeed, using the criteria described in this study might merely be detecting the normal cyclical fluctuations of rumen pH and thus also have a very low specificity for SARA. The cut-off point of pH ≤5.5 is a biological value, derived from normal rumen physiology and pathophysiology (Krause and Oetzel, 2006). Altering a threshold value can be used to increase specificity, and this option could be explored for SARA. It can be considered that in herds where a high proportion of cows have low rumen pH's for long periods of time, there will be a greater chance of detecting low pH at a single time point: hence the diagnosis of SARA using rumenocentesis and pH measurement should be reserved for herd level diagnosis rather than individual cow diagnosis.

However, if all cows in a herd reach their pH nadir at a similar time, and cows at peak yield are sampled (those eating the largest meals), then herd level diagnosis using single time sampling still remains a problem: an instance where a high proportion of sampled cows are ≤ pH 5.5 may merely indicate successful timing of sampling in relation to feeding to coincide with the nadir.

It is precisely because of these difficulties in reaching a diagnosis of SARA that some authors have questioned the use of the term at all. Calsamiglia et al (2012) recommend the use of the term "high concentrate syndrome", which also recognises that some of the clinical signs attributed to SARA are less likely to be from ruminal acidosis per se, but from large intestinal fermentation of excess dietary carbohydrate. This would concur with the work of Plaizier et al (2012) which suggests that the effects of SARA may be different depending on whether it is grain induced or low fibre induced. Kleen (2012) has also questioned whether the symptoms which clinicians often attribute to SARA are in fact more a signal of sub-optimal management in high yielding herds (which leads to the questioning of SARA as a clinical syndrome at all).

The rationale behind the "rumen health visits" during which the data for this study were collected, is to score various clinical attributes, each of which might give some clues to the management of the herd as well as the adequacy of nutrition. The visit includes scoring dry cows and pre-calvers, although none of these animals undergo rumenocentesis and so their data are not included in the study. A certain amount of value is attributed to the rumen protozoal scores, on the assumption that these might give a better indication of rumen health than a pH measurement which represents a single time point. For example, a cow might have suffered rumen acidosis in the preceding days, but whilst her pH might have recovered quickly due to the subsequent reduction in feed intake, the protozoa numbers would possibly still be affected. Likewise, a cow might still have good protozoa numbers if pH dips below the 5.5 threshold only transiently. Protozoa score is thus possibly a more stable variable than pH, and a more reliable indicator of rumen health.

Rumen pH undoubtedly affects the rumen micro-biome population and function (Plaizier et al, 2008; Khafipour et al, 2009c; Hook et al 2011a and 2011b, Poulsen et al, 2012), and protozoa represent a significant part of the rumen micro-biome. This study confirms that protozoa numbers and activity are affected significantly by rumen pH, concurring with previous findings by Kleen et al, (2009). Complete detection of the change patterns in the rumen microbial community during SARA may help in finding new methods for SARA detection in the future (Tajik and Nazifi, 2011). Factors other than pH, however, also affect the bacterial and protozoal structure in the rumen, including host and micro-biome inter-relationships which might be genetically pre-determined (Li et al, 2009). Hook et al (2011a) found that

recent water consumption can significantly affect protozoal concentrations. Protozoa concentrations could also be affected by in-feed ionophores (Russell and Strobel, 1989), such as monensin, used in some countries as growth and production promoters (but currently illegal in UK). In practice, temperature of the sample greatly affects the protozoal motility, so can affect the score attributed. The score is subjective, therefore, a protozoa score system as described here has some drawbacks for use as a SARA test, and for the reasons described above is not likely to be specific.

Comparing SARA diagnosis using rumen fluid pH measurement and diagnosis using low protozoal scores showed a significant correlation (Chi-square 63.47, $P<0.01$). Multivariable logistic regression showed that per unit increase in protozoal score, there was a significantly decreased risk of low rumen pH (≤5.5) (odds ratio 0.21, p-value <0.05). Using low protozoal scores as an alternative test for SARA showed better correlation with some of the clinical indicators (correlation with diarrhoea and low BCS became significant), but still with weak agreements (positive and negative predictive values). It cannot be deduced that low protozoal scores is a more reliable test for SARA than rumen pH, but there are some biological reasons for it conferring possible advantages.

Clinical indicators to diagnose SARA

The results show that commonly used clinical indicators for diagnosing SARA in herds or individuals, such as milk fat depression, low fat:protein ratios (at a recording ≤ 7 days from sampling), diarrhoea, excess long fibres in dung, and thin, empty (low rumen fill) cows are not reliable. This study also failed to show any association with ketosis, as measured by serum BHB. The overall prevalence of subclinical ketosis of 11.2% found in this study is in line with findings of a previous UK study into SCK prevalence of 9.4% in cows 5-60 days into lactation (Copper, 2011).

In fact, cows with the highest yields and fullest rumens had the lower pH values: this might be contrary to an archetypically described low yielding, low rumen fill cow suffering from SARA. This is therefore worthy of note and further investigation.

Although BCS did not correlate to rumen pH, there was a weak but significant (p-value 0.014) correlation between thin cows (BCS ≤2) and those with low protozoa scores (≤1). Kleen et al (2009)

found that SARA cows were more likely to have lost body condition in early lactation than non SARA cows, but the numbers of cows included in that analysis were very small. Presumably, cows with SARA are more likely to be in negative energy balance because of reduced ability to meet their energy requirements from efficient ruminal fermentation.

A similar weak correlation occurred between cows with diarrhoea (FC ≤2) and low protozoa scores (p-value 0.041). There were no correlations for cows diagnosed with SARA by pH measurement for any of the clinical indicators.

Faecal characteristics are commonly used in practice by nutritionists, vets and farmers to diagnose SARA (personal observation), although there is very scant published evidence to support this. The findings of this study indicated that faecal characteristics were not reliable predictors of rumen pH (or protozoa scores) supporting earlier findings by Kleen (2004).

Although milk fat depression is often used to diagnose SARA, there is scant evidence to support its use (Enemark, 2008, Iqbal et al, 2012). Its specificity and sensitivity is likely to be poor for the following reasons: inherent errors in measuring individual milk fats (Oetzel, 2007); milk fat being raised in conditions of rapid body fat catabolism in negative energy balance (Kleen et al, 2003, Plaizier et al, 2008); milk fats being affected by addition of dietary fatty acids, particularly unsaturated fatty acids which can depress milk fat (Enemark, 2008), or feeding rumen-protected fats which can increase milk fat (Doreau and Chilliard, 1997). Enemark (2008) suggests that more frequent individual milk fat analysis (weekly or daily as opposed to monthly) might increase the diagnostic value. Certainly, in this study, the milk fat was not necessarily tested on the day of rumenocentesis (the criteria for cows included in analysis was for cows to have a milk test within 7 days, either side of the rumenocentesis date). There might be a lag between low pH on one day, and occurrence of milk fat depression. However, low pH at the time of sampling presumably puts that cow at higher risk of SARA, which might be for an extended period. In addition, clinical experience suggests that milk fat values tend to remain fairly consistent for an individual from one consecutive recording date to another. This study adds to previous evidence (Tajik et al, 2009) that individual milk fat results cannot reliably be used to predict rumen pH or diagnose SARA.

Conclusions

- The prevalence of rumen pH ≤5.5, indicative of SARA, amongst 244 cows in a cross-sectional convenience sample of dairy cows in the central region of the UK was 26.2%
- Low rumen pH (SARA) could not be predicted using faecal characteristics, body condition score, rumen fill score, blood beta-hydroxybutyrate concentration, or milk fat percentage.
- Rumen protozoa (numbers and activity) were correlated to rumen pH: microscopic examination of rumen fluid for protozoa numbers and activity offers some scope for improving assessment of rumen acidosis, and rumen health, but further research is needed.

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Appendices

Appendix A

Rumenocentesis technique

The rumenocentesis site is on the cow's left hand side, at the level of the stifle, 2cm caudal to the last rib. Cows were restrained in a stall or a cattle crush which allowed access to the left flank. Another person holding the tail up assisted in keeping the cow still. The site was clipped (to mark it) and 3 mls of local anaesthetic (procaine) injected under the skin using a 1" 18 gauge needle. The site was not surgically prepared. Condition scoring the cow, assessing rumen fill and collecting the faeces and blood samples allowed sufficient time for the local anaesthetic to take effect.

A 16gauge 10cm needle (Air-Tite Products, USA) was used to aspirate approximately 5-10 mls of rumen fluid. The needle was advanced in a confident manner in a single, swift stroke aimed slightly cranially. The discomfort to the cow was minimal, but if the needle was advanced too gingerly, the abdominal muscles were more likely to twitch which was deemed more likely to cause damage to the rumen wall by the tip of the needle while it was advanced.

Appendix B

Protozoa Score descriptions

Microscopical inspection of rumen fluid was used to assess protozoal numbers and mobility. The rumen fluid was examined under low magnification (x40) using an illuminated dissecting microscope over a slide and plastic coverslip marked with a score line. Approximately 0.25 ml of warmed fluid (37 ±2 ℃) was placed under the coverslip and immediately examined using a subjective assessment with a simple scoring system to record results:

Table 11: Description of protozoal scores

Description	Protozoal score, PS
Highly motile and very crowded: a dense field of small and large protozoa darting randomly over the field of view. Large protozoa were uncountable as they transected a 1 cm line on the coverslip. Movement visible to naked eye.	+++ (3)
Motile and crowded, a mixture of small and large protozoa. Large protozoa were countable as they crossed a 1 cm transect line on the coverslip.	++ (2)
Sluggish motility and low numbers, mainly small protozoa. Large protozoa in a 1 cm diameter field of view were countable.	+ (1)
No or sporadic live fauna: < 2 large protozoa detectable in a 1 cm diameter field of view.	0

(Adapted from Atkinson, 2009)

Appendix C

Rumen Fill Score descriptions

Rumen fill scores were assessed by standing on the left hand side of the cow and looking at the paralumbar fossa, the triangle formed by the last rib (cranially), the transverse processes (dorsally) and the ilium (caudally). Scores 1 to 5 were recorded:

Table 12: Description of rumen fill scores

Score	Description
1	Empty rumen. The fossa is concave for more than a hand's width behind the last rib, and cavitates more than a hand's width under the transverse process. The fossa looks rectangular rather than triangular.
2	The fossa cavitates for a hand's width behind the last rib, and also beneath the transverse process. The fossa looks like a triangle. This score indicates an empty rumen.
3	The paralumbar fossa cavitates behind the last rib, but less than a hand's width, and the skin falls vertically from the transverse processes for around a hand's width before bulging outwards. This is the desired score for lactating cows with adequate dry matter intakes.
4	There is no concavity behind the last rib, and the rumen bulges immediately below the transverse processes. Dry cows and cows in late lactation should show this score, not because the rumen is more full, but the increasing size of the uterus in late gestation will make it appear so.
5	The rumen obliterates the fossa, and the last rib and ends of the transverse processes are not discernible. This score is only normal in heavily pregnant cows.

(Adapted from Atkinson, 2009 and Hulsen, 2012)

Appendix D

Faecal Consistency Score descriptions

Faecal consistency (FC) was assessed using fresh faeces only. Scoring is perhaps easier when the faeces is on a concrete floor, but a score was allocated when faeces was collected directly from the rectum for later sieving. Scores were 1 (thin) to 5 (firm):

Table 13: Description of faecal consistency scores

Score	Description
1	Watery thin. Faeces runs through fingers, and forms a liquid puddle on the ground, over a large area.
2	Faeces have a custard like consistency. On the ground, the faeces splashes over a large area and forms a pool, approximately 1 cm deep.
3	Faeces have a porridge like consistency. When dropped on the ground, the faeces makes a plopping noise and makes a thin, wide based pat, around 2cm thick.
4	Faeces have a consistency like thick porridge. If thrown at a wall, it would stick. When dropped it makes a heavy plopping noise and forms a well circumscribed pat with concentric rings like a large rosebud.
5	A firm faeces redolent of horse droppings.

(Adapted from Zaaijer and Noordhuizen, 2003)

Appendix E

Faecal (fibre) Digestion Score descriptions

Faecal (fibre) digestion scores were allocated after sieving a handful of faeces, the size of a tangerine, through a kitchen sieve with 1.75mm appertures. The faeces were sieved under a slow flowing faucet of water, and gently massaged around the sieve for around 30 seconds. The remaining particles in the sieve were then examined to give a score of 1 (well digested) to 5 (poor digestion):

Table 14: Description of faecal digestion scores

Score	Description
1	The faeces has a creamy, homogenous, well digested consistency and will pass through the sieve so that only a very small volume remains (less than 25% of original volume). The fibre that remains will be short length and fluffy. If rapeseed meal is fed, the small black seed husks will form part of this remaining fibre.
2	Less than a third of the faeces volume remains in the sieve. The remaining fibre is mainly short length but some larger, undigested fibre particles are present.
3	The faeces shrinks to only half its original volume. There are some lengths of fibre more than 1cm long. There may be a few incompletely digested concentrate particles, or maize grains.
4	The faeces shrinks to around 75% of its original volume. The remaining fibre is rough in texture, and some is more than 2cm long. There may be several undigested grains.
5	The faeces hardly shrinks in volume. The remaining fibre is rough and has the appearance of a TMR ration, with a lot of long (>2cm) fibre and undigested grains. There may be casts of intestinal mucosa present.

(Adapted from Atkinson, 2009, based on a method by Mgbeahuruike, 2007)

Appendix F

Cut-off thresholds for analysis and categories

In order to create binary categories of cows for various parameters, a cut-off threshold had to be used for cows with subclinical ketosis (SCK), thin cows, cows with empty rumen, cows with diarrhoea, cows with poor faecal fibre digestion, cows with with low milk butterfat and cows with a low milk fat: protein ratio.

Sub-clinical ketosis: there is no universally accepted definition for sub-clinical ketosis, but the majority of studies set a threshold of serum beta-hydroxybutyrate concentration of ≥ 1.2mmol/L being indicative of SCK (Cooper, 2011). This threshold was used in this study.

Thin cows: the recommended target condition score for lactating cows is 2.5, with ≤ 2 considered too thin (DairyCo, 2012).

Diarrhoeic faeces: Zaaijer and Noordhuizen (2003) recommend that lactating cows have a faecal consistency of 3; ≤ 2 is considered too liquid.

Faecal fibre scores: there are no recommendations for faecal fibre levels using the scoring system used, but Zaaijer and Noordhuizen (2003) described an alternative scoring system based on palpation of faeces through the fingers, and suggest that the ideal situation for lactating cows is where the faeces is homogenous with no undigested particles or fibres palpable. This would be analogous to scores 1 and 2. A score of ≥ 3 was considered undesirable, and was the cut-off point used in this study.

Rumen fill scores: Zaaijer and Noordhuizen (2003) suggest that the ideal score for lactating cows is 3. A score of ≤2 was used as the cut-off point in this study as being undesirably empty. A score of ≤2.5 was used in the multivariable logistic regression model, as RFS at this threshold had the higher odds ratio for risk of low pH (≤5.5).

Milk butterfat: there is no standardised butterfat percentage which is considered too low, or indeed indicative of acidosis. Husband (2005) suggested that a high proportion of cows in the first two months of lactation with butterfat <2.5% could be used as an indicator of acidosis. This threshold was tested in the study, but as only 2 cows were below this threshold, an alternative threshold of <3.5% was also used: this was the lower quartile of all cows.

Milk fat:protein ratios: there is no standardised interpretation of this ratio, but it has been observed that normally cows will have greater butterfats than protein (%), and when this proportion is reversed (BF/P <1), subacute ruminal acidosis could be a factor (University of Edinburgh Dairy Herd Health and Productivity Service, 2012). A butterfat/protein ratio of <1 was used as the threshold in this study.

Appendix G

2 x 2 contingency table to evaluate a test against a "gold standard"

To compare a value of a test under evaluation where the result is binary, the test positives and negatives can be compared against a "gold standard", as shown in table 15:

Table 15: An example of a 2x2 contingency table

		Condition determined by "gold standard" test		
		condition +ve	condition -ve	Total
Outcomes using test under evaluation	test outcome +ve	True positive a	False positive b (Type I error)	a+b
	test outcome -ve	False negative c (Type II error)	True negative d	c+d
	Total	a+c	b+d	a+b+c+d

Test characteristics can be calculated as follows:

Sensitivity: true positive result as a proportion of all positive conditions (= a/(a+c))

Specificity: true negative as a proportion of all negative conditions (= d/(b+d))

Positive predictive value: true positive results as a proportion of all test positive outcomes (= a/(a+b))

Negative predictive value: true negative results as a proportion of all test negative outcomes (= d/(c +d))

Accuracy: the proportion of results that are true (= (a+d)/ (a+b+c+d))

Positive and negative predictive values can only be estimated using data from a cross-sectional study or other population-based study in which prevalence estimates may be obtained. In contrast, sensitivity and specificity can be estimated from case-control studies, as they are independent of prevalence.

Kappa: this is a modification of the method by Landis and Koch (1977) to determine the level of agreement between two observers of categorical data. It can be used to compare agreement between two tests giving categorical results, to account for the level of "chance" agreements that might exist.

Test accuracy, (Pr a) = (a+d) / (a+b+c+d)

Expected "chance" agreement, (Pr e) = (a+c) x (a+b) / 2 x (a+b+c+d)

Kappa = (Pr a) - (Pr e) / 1 - (Pr e)

An alternative expression is:

$$\text{kappa} = \frac{\text{observed agreement - expected agreement}}{\text{total observed - expected agreement}}$$

A qualitative interpretation of the kappa value can be used as shown in table 16, but this is less relevant when comparing two tests than for two observers. The calculation can be used predominantly to allow ranking of tests in order of agreement.

Table 16: kappa values and interpretation, when used for two observers

Kappa	Interpretation
<0	no agreement
0.0-0.19	poor agreement
0.2-0.39	fair agreement
0.4-0.59	moderate agreement
0.6-0.79	substantial agreement
0.8-1.0	almost perfect agreement

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