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The possible role of transmissible gastroenteritis virus (TGE) and porcine respiratory corona virus (PRCV) in the mortality of Ugandan back-yard piglets

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Till Johanna

med kärlek och tacksamhet

Contents

1 Abstract

In Uganda, small scale pig production plays a major role for households to earn a living and to secure their access to meat. Piglet mortality due to diarrhoea-related dieseases is high. In an attempt to understand the underlying causes for this high mortality this study aims to investigate the role of transmissible gastroenteritis virus (TGEV) and is a part of a larger project conducted at Makerere university in Uganda.

A complicating factor in this investigation is the porcine respiratory coronavirus (PRCV), a stabile mutant form of the TGE virus. The occurrence of this virus had shown to alter the impact of TGEV in e.g. Europe and North America. The interaction between these two viruses is discussed. Even porcine epidemic diarrhoea (PED) is a coronavirus-induced disease of swine and reports on the presence of all three viruses in the world is reviewed.

48 serum samples from adult pigs from the district of Gulu in Uganda were tested. The test used in this study is an enzyme-linked immunosorbent assay (ELISA) which is able to identify both TGEV and PRCV and to differentiate between these viruses. Test results for all samples showed to be negative. The result is discussed by considering a possible impact of the extensive heat-inactivation on the samples as well as geography, climate and trade pattern of the district of Gulu in Uganda.

2 Sammanfattning

I Uganda har grisproduktion i liten skala stor betydelse för att hushållen ska kunna få en inkomst och säkra sin tillgång till kött. Spädgrisdödligheten på grund av diarrésjukdomar är hög. I ett försök att förstå de underliggande orsakerna för denna höga dödlighet avser denna studie att undersöka betydelsen av transmissible gastroenteritis virus (TGEV) som en del av ett större projekt genomfört av Makerere universitetet i Uganda.

En komplicerande faktor i denna undersökning är porcine respiratory coronavirus (PRCV), en stabil muterad form av TGE viruset. Förekomsten av detta virus har visat sig kunna förändra påverkan av TGEV i t.ex. Europa och Nord Amerika. Interaktionen mellan dessa två virus diskuteras. Även porcine epidemic diarrhoea (PED) är en coronavirus-inducerad grissjukdom och rapporter om förekomsten av dessa tre virus i världen återges.

48 serum prover från vuxna grisar från Gulu-distriktet i Uganda testades. Testet som användes i denna studie är en enzyme-linked immunosorbent assay (ELISA) som kan identifiera både TGEV och PRCV och skilja mellan dessa två. Testet visade negativa resultat för samtliga prov. Resultatet diskuteras med hänsyn till en möjlig påverkan av den omfattande värmeinaktiveringen av proverna men även geografi, klimat och handelsmönster i Gulu-distriktet i Uganda.

3 Introduction

3.1 Background

In Uganda, small scale pig production is a common means for families to secure access to meat and to provide an income (Nissen et al. 2011). Thus, owning pigs does not only improve a family`s economy through meat sales or as a savings instrument but even increases the social status of the household (Perry et al. 2002). In the countries of southern Africa it is often the women and children who own and take care of the animals (Chimonyo et al. 2005). Some of the Ugandan pigs are of Yorkshire-breed, imported to the country, but the majority of the pig population consists of indigenous breeds. Since the 1980s the pig population has increased tremendously in the whole country and the production of pig meat has grown more than tenfold from 1985 until 2009 (FAOSTAT 2011). Especially in the north and northeastern parts of the country, pigs seem to have served as a replacement for the cattle lost in the wake of the civil strife during the 1970s (FAO 2004). The conditions under which these animals are kept are very simple compared to the industrialized environment of intensive pig farming in e.g. Europe. Approximately 80% of all pigs are kept traditionally in Uganda, and the pig production of the developing countries relies to 70% on traditional production using mainly local breeds or cross-breeds (Lekule and Kyvsgaard 2003). Most of the pigs are kept by small household units (Ikwap 2011). The traditional ways of pig keeping in such small scale systems are free ranging, tethering or confinement in simple sties (Lekule and Kyvsgaard 2003). The breeding is usually accomplished by mating the sows to a boar and they are usually brought to the boar for that purpose. Such breeder boars naturally represent an important source for spreading infections among the sows and other nearby pigs

(Wahlström et al. 1990). The greater part of the litter is usually lost due to diarrhoea-related diseases and the dominating disease agents involved are yet to be identified (Ikwap 2011).

Livestock production in small scale enterprises and in the private sector is of vital importance in many developing countries, often contributing to a considerable extent to the countries` food production. These small production units not only face a greater exposure to risk through a greater presence of disease and less disease control compared to production in industrialized countries, but also their capacity to bear risk is lower due to the small size of their livestock and the lack of economic reserves. An increase in efficiency of these livestock-producing activities would lead to an increase in income at the very source of the production (Perry et al. 2002).

Pig farming in particular can be a very effective way to produce meat considering the fast reproduction cycle and the high feed conversion efficiency (Lekule and Kyvsgaard 2003). However, to achieve a high efficiency in pig farming in the described setting, one obviously crucial factor is to improve piglet survival. Therefore, an understanding of what are the most common fatal infectious diseases among piglets in Uganda is needed. At the University of Kampala, a PhD project currently focuses on the role of *E.coli* and *Salmonella* spp. for piglet mortality (Ikwap 2011). However, a broad spectrum of disease agents must be considered as possible differential diagnoses. In this context the present study aims to examine the prevalence of transmissible gastroenteritis virus (TGEV) as a possible agent for disease in the Ugandan pig population. TGEV is also an important differential diagnosis for *E. coli*-associated diarrhoea.

3.2 Coronavirus

Both TGEV and porcine respiratory coronavirus (PRCV) are of the genus *Coronavirus* which is a member of the family *Coronaviridae.* The family of *Coronaviridae* is in turn included in the order *Nidovirales* (Saif 2011). There is a great number of different pathogenic coronaviruses which cause a wide variety of diseases among mammals and birds. These pathogens are subdivided into three different groups and further into subgroups according to their genetic and serologic properties. TGEV and PRCV both belong to the same

group and subgroup, i.e. group 1a. Their virions, often spherical in shape, display large club-shaped spikes when seen under the electrone microscope (Saif 2011). The virions are enveloped and have an icosahedral internal core structure containing a helical nucleocapsid. Their surface exhibits a number of different structural proteins, surface glycoproteins, which the host immune system reacts to. Apparently, the neutralizing antibodies produced by the humoral immune response are mainly directed against the S protein (the major spike glycoprotein), whereas the cellular immune response targets both the S protein and the N protein (nucleocapsid protein). Further known structural proteins of the coronavirus are the transmembrane glycoproteins M and E and in some coronaviruses also hemagglutinin esterase, HE (Saif 2011). The S protein, which is necessary for cell attachment and, at the same time, constitutes the main antigenic component of the virion, has some hypervariable domains enabling the formation of virus-escape mutants. In this way coronaviridae are able to evade the immune response of the host (Quinn et al. 2011).

3.3 TGE and PRCV

Transmissible gastroenteritis (TGE) is a viral enteric disease that infects swine worldwide. It was reported for the first time in 1946. The disease is caused by a coronavirus called the transmissible gastroenteritisvirus (TGEV). In the 1980s a genetic variation of the TGE virus appeared, which was called the porcine respiratory coronavirus (PRCV) according to its tropism for the respiratory tract (Bohl 1989, Pensaert 1989, Saif and Sestak 2006).

The appearance of the porcine respiratory coronavirus (PRCV) in Europe and the USA in the 1980s has changed the impact of TGEV. PRCV is a stable deletion mutant form of the TGE virus and antibodies against TGEV cross-react with PRCV. Thus, infection with PRCV, leading to antibody production, also seems to be protective against TGEV infection to a certain extent. In those European countries and parts of North America where PRCV occurs endemically, the impact of TGEV has declined (Saif and Sestak 2006). In fact, antiserum produced against TGEV neutralizes PRCV completely at the same neutralization titer (Pensaert 1989). This poses a challenge to diagnostic

methods that have to be able to distinguish antibodies against PRCV from those against TGEV (Saif and Sestak 2006).

3.3.1 Epidemiology and pathogenesis of TGE

TGE is transmitted in a fecal-oral pathway. It appears in an epidemic and an endemic form. In its epidemic form it is found in naïve herds, i.e. where no seropositive animals are found. Usually, animals of all ages are affected and the disease spreads quickly. Typically, infections occur during the winter-time. The clinical signs vary with the age of the pigs. Piglets below 2-3 weeks of age will be struck hardest by the disease. The TGE virus destroys the intestinal epithelium of the small intestine thereby impairing its absorptive capacity. Vomiting, profuse, watery, yellowish diarrhoea containing undigested milk and, consecutively, loss of weight and dehydration occurs. The mortality rate for the youngest piglets is close to 100%. Piglets older than 3 weeks often survive, but growth will be impaired. Growing and finishing pigs as well as sows usually show limited signs of disease, inappetence, diarrhoea and vomiting being the most common. Lactating sows, though, can become very sick with concurrent agalactia affecting her infected litter even more. The endemic form of TGE is found in herds where the virus persists. This is only possible in herds where there is a steady inflow of seronegative animals or continuous farrowing. Endemic TGE often follows an epidemic episode of TGE in the herd. The infection spreads slowly in contrast to the epidemic scenario and immune sows mitigate the clinical picture through the passive immunity passed on to their suckling piglets via their colostrum. The piglets show varying degrees of clinical disease and mortality normally lies under 10-20% depending on their age at infection, the amount of maternal antibodies ingested and a number of other factors. In such cases it will be a challenge to differentiate endemic TGE from other causes for endemic diarrhoea such as rotavirus diarrhoea, colibacillosis or porcine epidemic diarrhoea (PED) (Saif and Sestak 2006).

3.3.2 Epidemiology and pathogenesis of PRCV

PRCV infect pigs through airborne and contact transmission. Pigs of all ages are susceptible. In places with high density of swine farms and large herds the virus can cause infection over distances of several kilometers. PRCV infection usually leads to milder disease with symptoms from the respiratory tract and the course of disease is often subclinical. These circumstances con-

tributed to a rapid spread of the virus in Europe and many other parts of the world, even countries that had not previously been affected by TGEV. In many swine herds in Europe PRCV is regarded endemic today (Saif and Sestak 2006).

3.3.3 Interactions between TGEV and PRCV

The fact that antibodies against PRCV cross-react with TGEV, in connection with the observation that the impact of TGEV in positive herds declined after the introduction of PRCV, suggests that a PRCV-induced immunological response could be protective against TGEV. This could be shown experimentally for neonatal pigs (Wesley and Woods 1996). In another trial Wesley & Lager showed that the PRCV-priming of female piglets could induce a secondary immune response to TGEV shortly before farrowing, resulting in a higher litter survival rate compared to the non-primed gilts (Wesley and Lager 2003). Bernard et al. could report on the protective ability of prior PRCV infection against TGEV through lactogenic immunity (Bernard et al. 1989). Still, a full protection against TGEV from PRCV infection cannot be expected *per se* (van Nieuwstadt et al. 1989, Bernard et al. 1989). The coronavirus is unstable by nature which claims for constant surveillance of the development of its different serotypes. Done mentions the need for further efforts to characterize the viruses since the protective properties of PRCV against TGEV may have decreased or TGEV might have become more virulent after its unexpected return in Great Britain in 1996 (Done 2000). Even Pritchard et al. call for the evaluation of the emergence of new variants of viruses (Pritchard et al. 1999).

3.4 PED – Porcine Epidemic Diarrhea

Another disease that is caused by a coronavirus is porcine epidemic diarrhoea (PED). The clinical signs strongly resemble those of TGE with watery diarrhoea being the most obvious, but the piglet mortality tends to be lower as compared to TGE, normally lying around 50%. In some cases, though, it may be as high as 100%. Apparently, severe outbreaks with extremely high mortality among newborn piglets were reported from Japan and Korea in recent years but have been rare in Europe. Signs of more pronounced abdominal pain and sometimes sudden deaths among adult and finisher pigs are clues that may help to differentiate PED from TGE. Probably, PED does not spread

as quickly between groups as TGE and it seems to be more persistent on a farm after the acute phase of an outbreak. Endemic infection is not unusual. The mode of transmission seems to be the same as for TGE, being mainly faecal-oral (Pensaert and Yeo 2006).

3.5 TGEV, PRCV and PED in the world

Until now, Sweden is considered free from TGEV and annual testing is undertaken to confirm this status. PRCV was found in south-western Sweden already in 1986 and had spread over the whole country by 1993. Since then there have been 2 incidences where boars had tested positive for TGEV. Further investigation could not confirm the presence of TGEV and it was assumed that the results were due to instable mutations of PRCV. The fact that coronaviruses constantly mutate supports the assumption that frequently arising mutation variants of PRCV induce antibodies that are able to undermine the results of the test by cross-reacting with TGEV. This causes problems since the necessary follow-up testing to exclude TGEV takes time and delays the work flow of the boar stations with high costs for the pig industry as a whole in consequence (Berndtsson et al. 2006). Since its discovery, TGEV has been reported from countries all over the world (Rodriguez Batista et al. 2005). In many European countries the prevalence of seropositive herds is almost 100% following the emergence of PRCV. Also in North America TGE is widely spread (Saif and Sestak 2006). TGE occurs both in a more silent endemic form and as clinically evident disease outbreaks. If these different appearances are linked to the presence or absence of PRCV infection is not completely clear (Pritchard et al. 1999). Also PRCV seems to be endemically spread in many European countries (Saif and Sestak 2006) with reports confirming its appearance in e.g. North America (Wesley et al. 1997), Japan (Miyazaki et al. 2010), Britain (Pritchard et al. 1999), Korea (Chae et al. 2000) and Italy (Martelli et al. 2008). Reports from the African continent have been rather scarce. In 1999, Uganda apparently became the first country in Africa that reported the presence of TGE (Rodriguez Batista et al. 2005). Though, according to OIE statistics Uganda did not report TGE for the year 2005 and for the following years until today no information has been available. It remains unclear if the 1999 report was based on erroneous test results or if the disease disappeared again. Other African countries that reported the disease as “suspected but not confirmed” since 2005 are the Central African

Republic (2007 and 2008), Rwanda (2010) and Togo (2009). Only The Democratic Republic of the Congo reported its TGE status as "confirmed infection but no clinical disease" for the years 2005, 2006 and 2007 (OIE 2011). Obviously, TGEV is present on the African continent but more detailed information on its prevalence in different African countries is not at hand. The prevalence pattern of PRCV and PEDV across the African countries also remains rather unknown.

3.6 Differential diagnoses to TGEV-diarrhoea and PED

There are of course many causes for diarrhoea in young piglets and many of the pathologic agents induce disease during specific periods. The list below shows the common diarrhoeic diseases in a chronological order (Jacobson 2003).

- Neonatal diarrhoea during the first week of life when the piglets' immunity depends completely on the maternal antibodies from the colostrum.
  - Colibacillosis (*E.coli*)
  - Transmissible gut gangrene (*Clostridium perfringens* Type C)
  - *Clostridium perfringens* Type A
  - Transmissible gastroenteritis (TGEV – Coronavirus) and porcine epidemic diarrhea (PEDV – Coronavirus) have the most severe impact on the animal during the early neonatal period.
  - New neonatal porcine diarrhoea (NNPD) with yet unknown etiology (Svensmark 2009).

- Diarrhoea during week 2 and 3 when the concentration of maternal antibodies has decreased and the production of own antibodies not yet fully developed.
  - Neonatal steatorrhoea (*Isospora suis* or rotavirus)
  - Secondary infection with *E.coli*

- Post-weaning diarrhoea during the first two weeks after weaning. Several predisposing factors such as changes in feed seem to play an important role during this period.
  - Enterotoxigenic *E.coli* (ETEC)

- About 2-3 months after birth.
  - Spirochetal diarrhoea (*Brachyspira pilosicoli*)
  - Proliferative enteropathy (*Lawsonia intracellularis*)

- Other diarrhoeic diseases with less pronounced connection to the pigs' age.
  - Transmissible gastroenteritis (TGEV – Coronavirus)
  - Porcine epidemic diarrhoea (PEDV – Coronavirus)
  - Swine dysentery (*Brachyspira hyodysenteriae)*
  - Salmonellosis (*Salmonella* spp., especially *S.* Cholerasuis)

During the past decade, also the Postweaning multisystemic wasting syndrome (PMWS) which is associated to the porcine circovirus type 2 (PCV2) has become a recognized etiology of diarrhoea in nursery and growing pigs (Segalés and Domingo 2002).

To what extent the listed disease agents are relevant in small scale pig production in the Ugandan setting is unknown, but this work may be a modest contribution to clarify the situation for at least TGEV/PRCV.

4 Materials and methods

4.1 Experimental design

The test used in this study is the SVANOVIR® TGEV/PRCV-Ab (Svanova Biotech AB, Uppsala, Sweden) which is able to detect and differentiate between TGEV- and PRCV-specific antibodies. Thus, an advantage with this test is that even the prevalence of PRCV could be detected which, as previously discussed, may alter the impact of a possible TGEV infection. The SVANOVIR® TGEV/PRCV-Ab is an enzyme-linked immunosorbent assay (ELISA).

4.1.1 Sample collection and processing

Blood samples were collected in 48 different households in Uganda in the district of Gulu. The households were usually situated several kilometers apart and a majority kept 1 to 3 adult pigs. Blood samples were collected from the jugular vein using sterile 21 gauge needles and sterile 4 ml vacutainers without anticoagulant (BD-Playmouth, UK). The samples were then put on ice and transported in a cool-box to the veterinary office of the district. Here, the samples were refrigerated at 4°C overnight so a clot could develop. The following day the serum was separated, filled into sterile screw-capped micro-tubes (Sarstedt, Germany) and transported on ice in a cool-box to the laboratory at the Makerere University in Kampala where it was kept at -20°C. A total of 60 samples were taken whereof 59 came from adult pigs and one from a 3 week old diarrhoeic piglet. Seven of these samples showed severe haemolysis and were retained, and one sample was lost. The remaining 52 samples were inactivated by heating at 56°C for 30 minutes and were then sent to the Swedish National Veterinary Institute (SVA) in Uppsala at 4°C.

Here, the samples were tested for Foot-and-mouth disease and African swine fever due to security routines. In this course 3 samples were identified as mildly positive for foot-and-mouth disease. These samples were removed from further analysis and the remaining samples were inactivated a second time, now at 70°C for 120 minutes. One other sample was removed because it did no longer contain any fluid. Finally, 48 samples, all from adult pigs, were used in the analysis. 14 of the samples were to a greater or lesser extent coagulated which made it difficult to retrieve the exact amount of fluid necessary for the test.

4.1.2 The ELISA test

The ELISA test has been described as a useful test for the detection of TGE (Hohdatsu et al. 1987). There are different kinds of ELISA tests which function in slightly different ways (Tizard 2004). The SVANOVIR® TGEV/PRCV-Ab used in this study is an indirect blocking ELISA test. The TGEV-specific viral antigens are attached to the wells of a microtitre plate. Thereafter, the serum sample is added in pairs, i.e. in two different wells. Any antibodies against TGEV or PRCV present in the serum sample will bind to the antigen on the plate, hence the term “blocking”. If no such antibodies are found in the serum sample the binding sites remain free (Fig. 1).

Then, a solution containing monoclonal mouse IgG antibodies against TGEV is added to the first well and another solution containing monoclonal mouse IgG antibodies against TGEV/PRCV to the second well. If the respective binding sites of the antigen are still free, i.e. if no antibodies against the disease agents were present in the serum samples, the monoclonal mouse-antibodies will attach to them. In the next step, enzyme-conjugated anti-mouse IgG antibodies against the former monoclonal mouse IgG antibodies are added. These can only bind to epitopes on the monoclonal mouse IgG antibodies. Since these epitopes are specific, unspecific binding of any swine-antibodies will not occur. If the serum is free from TGEV or PRCV antibodies, both the monoclonal mouse-antibodies, and in the next step the enzyme-conjugated anti-mouse IgG antibodies, will have bound to the plate. In a final step a substrate is added that reacts with the enzyme and induces a colour change.

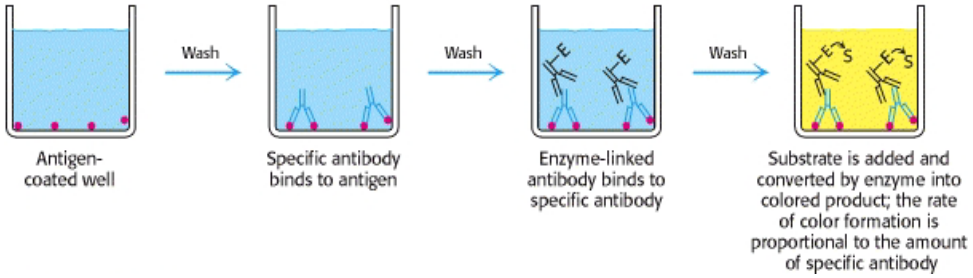

Figure 1 – The binding of mouse IgG antibodies and enzyme conjugated anti-mouse IgG antibodies to the antigen coated wells in the absence of specific swine antibodies against TGEV or PRCV in the serum sample (Chakravarthy 2011).

Therefore, a strong colour change indicates a negative result, i.e. there were no antibodies against the disease agent present in the sample. In the absence of a colour change the test result will be positive, since the antibodies against TGEV or PRCV in the serum sample will have blocked the binding sites.

A characteristic of the SVANOVIR® TGEV/PRCV-Ab is its ability to differentiate between TGEV and PRCV antibodies. The fact that naturally existing antibodies against PRCV are able to cross-react with TGEV is used in the test. The test kit contains one solution with mouse IgG antibodies against TGEV (Anti-TGEV mAb solution) and another solution with mouse IgG antibodies against PRCV which can also bind to TGEV (Anti TGEV/PRCV mAb solution). Each serum sample is added to a pair of wells, one for each of the two antibody solutions. The presence of swine TGEV antibodies in the serum samples will block the attached TGEV antigens in the wells of the microtitre plate for both the mouse antibodies against TGEV added to the first well, and for those against TGEV/PRCV added to the second well. Instead, swine PRCV antibodies in the serum samples will only block the attachment of the mouse antibodies against TGEV/PRCV added to the second well, leaving the site for the mouse antibodies against TGEV in the first well free. Logically, after the application of (1) the swine serum samples, (2) the respective mouse mAb solutions and (3) the enzyme conjugated anti-mouse antibodies, the test results must be interpreted in the following way (Table 1):

Attachment of mouse TGEV mAb in the first well containing sample x	**Attachment of mouse TGEV/PRCV mAb in the second well containing sample x**	**Interpretation of the combined results from well 1 and 2**
Blocked (no colour change)	Blocked (no colour change)	TGEV+
Not blocked (colour change)	Blocked (no colour change)	PRCV+/TGEV-
Not blocked (colour change)	Not blocked (colour change)	PRCV-/TGEV-

Table 1 - Interpretation scheme for laboratory results

In the first scenario, the blocking of both mouse TGEV mAb and mouse TGEV/PRCV mAb could have been caused by the presence of swine TGEV antibodies alone but it does not automatically exclude the presence of swine PRCV antibodies. Therefore, the result can only be interpreted as TGEV+ with certainty and the presence of swine PRCV antibodies can neither be excluded nor confirmed (Svanova Biotech AB 2011).

4.1.3 Quantification of the results and validation of the test

To quantify the color change and to be able to relate it to the positive and negative controls, the optical density (OD) is measured with a photometer at 450 nm. Naturally, the negative control is supposed to give the greatest possible color change (maximum OD). The following formula is used to calculate the percent inhibition (PI), i.e. the deviation of the OD of the sample from the greatest possible color change, namely the OD of the negative control, in relation to the OD of the negative control:

$$PI = \frac{OD_{neg\ ctrl} - OD_{sample}}{OD_{neg\ ctrl}} \times 100$$

In the presence of a high concentration of blocking swine-antibodies in the serum the OD of the sample will be low, resulting in a high PI. In turn, the absence of any swine antibodies will result in a high OD showing a low PI. Test validity is examined by the application of control sera. The cut-off values for test validity according to the manufacturer are the following:

- Negative TGEV/PRCV Control Serum: OD > 0.5
- Positive TGEV Control Serum with Anti-TGEV mAb: PI > 60
- Positive TGEV Control Serum with Anti-TGEV/PRCV: PI > 60
- Positive PRCV Control Serum with Anti-TGEV mAb: PI < 15
- Positive PRCV Control Serum with Anti-TGEV/PRCV: PI > 60

The cut-off values for the interpretation of the results from the serum sample analysis are:

- PI > 60 - positive
- PI 45-60 – doubtful
- PI < 45 – negative

4.1.4 Sensitivity and specificity of the test

The SVANOVIR® TGEV/PRCV-Ab has been tested at herd level and was found to provide a diagnostic sensitivity of 0.933 (95% confidence interval: 0.779, 0.992) and a diagnostic specificity of 0.943 (95% confidence interval: 0.86, 0.984) for the detection of TGEV which was considered good. It shall be mentioned that the test achieved a sensitivity of 100% for TGEV in experimentally infected pigs 21 days after the day of infection. Before that, the test did not show consistently correct results. The ability of the test to diagnose animals infected early during an acute outbreak is therefore limited (Carman et al. 2002). No information could be found on the sensitivity or specificity of the test regarding the detection of TGEV in individuals or the detection of PRCV in general.

4.2 Laboratory analysis procedure

The manual of the SVANOVIR® TGEV/PRCV-Ab test kit was followed in all details. Briefly, the procedure included the following steps:

1. The plates were washed with PBS-Tween buffer 3 times according to the instructions.
2. 100 µL each of the different control sera were added to the wells.
3. 50 µL of the serum samples together with 50 µL of PBS-Tween were added in duplicates to the wells.
4. Incubation for 2 hours at +37°C.
5. Step 1 was repeated.
6. 100 µL of Anti-TGEV Solution was added to the first of the sample duplicates and 100 µL of Anti TGEV/PRCV was added to the second of the sample duplicates. Incubation for 30 minutes at room temperature.
7. Step 1 was repeated.
8. 100 µL of diluted HRP Conjugate Solution was added to the wells. Incubation for 30 minutes at room temperature.
9. Step 1 was repeated.
10. 100 µL of Substrate Solution was added to the wells. Incubation for 30 minutes at room temperature.
11. 50 µL Stop solution was added to the wells to stop the reaction.
12. The OD was measured at 450 nm in a spectrophotometer within 15 minutes after the application of the Stop Solution.

5 Results

5.1 Controls

All controls, both on the first and on the second microtitre plate indicated test validity. The test values for the controls are shown in the table below.

Control serum	mAb solution	Plate 1	Plate 2	Cut-off value
		Percent inhibition		
Pos. TGEV	Anti TGEV	+ 74.8	+ 70.0	PI > 60
Pos. TGEV/PRCV	Anti TGEV/PRCV	+ 83.7	+ 79,4	PI > 60
Pos. TGEV	Anti TGEV	- 0.1	- 0,3	PI < 15
Pos. TGEV/PRCV	Anti TGEV/PRCV	+ 90.3	+ 87,2	PI > 60
		Optical density		
Neg. TGEV/PRCV 1	Anti TGEV	1.487	1.039	OD > 0.5
Neg. TGEV/PRCV 1	Anti TGEV/PRCV	1.312	1.054	OD > 0.5
Neg. TGEV/PRCV 2	Anti TGEV	1.527	1.000	OD > 0.5
Neg. TGEV/PRCV 2	Anti TGEV/PRCV	1.383	1.013	OD > 0.5

Table 2 – Results of Control sera

5.2 Serum samples

After measuring the optical density (OD) of each well the percent inhibition (PI) of each sample could be calculated. A cut-off value of less than 45 for the percent inhibition (PI) was indicative of a negative result and values within the range of 45 to 60 were regarded doubtful. The calculated values for the PI of the analysed samples ranged from -11.8 to 24.2 (See Table 1 in the appendix for the values of each sample). These values clearly indicate negative results. This means that neither antibodies against TGEV nor against PRCV could be detected. No samples were regarded as doubtful.

6 Discussion

6.1 Laboratory method

Since it is the aim of this study to either confirm or reject the presence of a specific pathogen among a number of other possible pathogens that cause diarrhoea in piglets, the method used must be able to reliably identify TGEV. A diagnosis by clinical signs is therefore impossible. A biochemical diagnostic method is a more appropriate tool. The collection of samples had to be done in the field and therefore it would have been difficult to time each sample collection with an outbreak of diarrhoea among piglets. This would have been necessary because the detection of TGEV antigen requires that the samples are taken in an early stage of the disease (Saif and Sestak 2006). Another possibility would be to detect antibodies against TGEV. One advantage is that antibodies will be present in the blood for a long time after infection (Saif and Sestak 2006). This makes sample collection much easier. Even if the pathogen itself is not found, the presence of antibodies can be taken as a proof for previous infection and suggests an endemic TGEV status.

6.2 Epidemiologic approach

When individuals that do not show any clinical signs are tested for the purpose of detecting a disease in the population, the measure is called a screening. From a clinical perspective, early diagnosis achieved through a screening measure should help to treat the condition more efficiently in order to make the screening worthwhile (Piantadosi 2005, Dohoo et al. 2010, Petrie and Watson 2006). The purpose of the screening performed in the present study is rather the attempt to identify a history of infection with TGEV among the

sows tested. The outline of this cross-sectional study was rather modest and a more sophisticated design of the study using e.g. paired samples would have been desirable to be able to more precisely determine the time of a possible infection and thereby the probability for the disease´s impact on the pathogenesis. Still, considering that TGEV was not the main focus of the project and the scarcity of the resources, the study does contribute valuable information. It will be one piece in a puzzle which in the end may give a picture of the spectrum of disease agents that actually contribute to Ugandan piglet mortality. Only then the question of efficient treatment or appropriate management measures can be addressed.

6.3 Interpretation of the results

The interpretation of the negative result for all 47 samples is not straight forward. With regard to the quality and the treatment of the test material the possibility of false results needs to be discussed.

6.3.1 Coagulation

14 of the samples were marked as problematic with regard to their quality. In these cases it was either impossible or so difficult to receive the required amount (50 µL per well) of serum from the sample that the test operator decided to make a remark. The coagulation/aggregation of serum may lead to a mechanical entrapment of particles, such as antibodies. This can prevent antibodies from binding to the antigens attached to the plate consequently showing false negative results. A suggested practice to avoid this problem is to mix the clot with buffer solution and then dissolve it mechanically as well as possible. After centrifugation, the supernatant can be used as sample. In this way, the mechanical entrapment of antibodies present in the clot could be dissolved (M. Merza, personal communication).

6.3.2 Heat treatment of the samples

Heat inactivation of serum samples appears not to be regarded as harmful to antibody structure, in particular IgG. However, little scientific proof has been found to support this common opinion (Reynolds et al. 1977). Heat inactivation should still be considered as a possible source of false results since the

heat stability of immunoglobulin is not undisputed (Fan et al. 2009, Chantry et al. 2009). The process of denaturation of IgG appears to be complex with changes of e.g. the secondary structure occurring at 60°C and 70°C, respectively. Depending on the temperature and the rate of the heating, the possibility of protein aggregation before the complete unfolding of the proteins also must be considered (Vermeer and Norde 2000). In the field testing of the SVANOVIR® TGEV/PRCV-Ab for repeatability of the results after heat inactivation of the serum samples, a temperature of 56°C for a period of 30 minutes was applied which did not affect the results negatively (Carman et al. 2002). This is below the temperature that was used for the samples in this study and therefore it is difficult to discuss the possible heat treatment effects.

6.4 Conclusion

Even if the presence of TGEV/PRCV among the pig population in the district of Gulu in Uganda cannot be ruled out with certainty, the test results may still be interpreted as a hint to the absence of TGEV/PRCV. The fact that Uganda has reported TGEV in 1999, but never since, is staggering and must be evaluated in the light of test results and observations done before and after the report. Most of the quality issues regarding the samples could probably be solved by conducting the ELISA-testing in close connection to the sample collection. If the testing would be done at the laboratory of the Makerere University in Kampala the extensive heat-inactivation procedures that were undertaken in Sweden would not be necessary. The test could also be used more specifically in outbreak situations where a suspicion of TGEV or PRCV infection arises. All this would contribute to more reliable test results increasing their usefulness. Therefore, it would be desirable that the colleagues in Kampala could operate the ELISA test used in this study themselves. If such a testing on site also would show negative results, confirming the absence of TGEV/PRCV, further studies could identify the entrance of TGEV/PRCV into the country if this was desirable.

6.5 Outlook

The prevalence and the impact of TGEV depend on various factors. Concurrent presence of PRCV is obviously one that can mitigate the impact of

TGEV. Other factors that might play a role may be trade, transport and climate. TGEV is described as sensitive to sunlight and warm climate. This may affect the virus` survival in the Ugandan countryside. In many parts of Africa there is no extensive trade or transport of animals such as pigs. Instead, production, trade and consumption are often rather local. This may be an effective barrier for the diseases, especially TGEV which is transmitted by a fecal-oral pathway. One obvious question is, of course, how a changed trade and transport pattern would alter the situation. The fact that TGEV has been reported from the neighboring Democratic Republic of Congo and the increase in pig production in Uganda, even in a more industrialized manner, makes this question a less hypothetical one. Even if the district of Gulu is rather separated from the Democratic Republic of Congo by the Nile, the southwest of Uganda could be a potential path of entry for TGEV and might be worthwile to assess. In case of the establishment of TGEV in Uganda, several questions would arise, such as what impact the introduction of PRCV would have on the severity of TGEV outbreaks. Would PRCV decrease the severity of TGEV infection in the same way as it seems to have done in many other countries? The propensity to mutation of the coronavirus could lead to new variants (Pritchard et al. 1999) of TGEV or PRCV which might not show the same pattern of interaction. Another unreviewed study which was conducted in Hungary even suggests that it might not be the genetic properties of these viruses but rather the immune response of the pigs that play a role in the re-emergence of TGE in some countries. The author mentions the possibility that the widespread porcine circovirus (PCV) with its immunosuppressive properties may inhibit antibody production against PRCV and TGEV (Andersson 2010). Vaccination against TGEV is possible though not fully protective (Tuboly et al. 2000). Besides, it is costly and may therefore be an unrealistic measure in Ugandan backyard pig production especially with regard to African swine fever and foot-and-mouth disease being actual identified threats in the region.

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Appendix

Table 1 – Results of the ELISA test

Sample no.		A OD neg. Control	B OD sample	A-B	((A-B)/A)*100 Percent Inhibition
1	TGEV	1,42725	1,355	0,07225	5,062182519
1	TGEV/PRCV	1,42725	1,364	0,06325	4,431599229
2	TGEV	1,42725	1,473	-0,04575	-3,205465055
2	TGEV/PRCV	1,42725	1,499	-0,07175	-5,027150114
3	TGEV	1,42725	1,392	0,03525	2,469784551
3	TGEV/PRCV	1,42725	1,43	-0,00275	-0,192678227
5	TGEV	1,42725	1,364	0,06325	4,431599229
5	TGEV/PRCV	1,42725	1,428	-0,00075	-0,052548607
6	TGEV	1,42725	1,473	-0,04575	-3,205465055
6	TGEV/PRCV	1,42725	1,402	0,02525	1,769136451
8	TGEV	1,42725	1,478	-0,05075	-3,555789105
8	TGEV/PRCV	1,42725	1,359	0,06825	4,781923279
11	TGEV	1,42725	1,595	-0,16775	-11,753371869
11	TGEV/PRCV	1,42725	1,345	0,08225	5,762830618
12	TGEV	1,42725	1,432	-0,00475	-0,332807847
12	TGEV/PRCV	1,42725	1,274	0,15325	10,737432125
13	TGEV	1,42725	1,148	0,27925	19,565598178
13	TGEV/PRCV	1,42725	1,222	0,20525	14,380802242
15	TGEV	1,42725	1,467	-0,03975	-2,785076195

15	TGEV/PRCV	1,42725	1,39	0,03725	2,609914171
17	TGEV	1,42725	1,472	-0,04475	-3,135400245
17	TGEV/PRCV	1,42725	1,264	0,16325	11,438080224
18	TGEV	1,42725	1,434	-0,00675	-0,472937467
18	TGEV/PRCV	1,42725	1,378	0,04925	3,450691890
19	TGEV	1,42725	1,459	-0,03175	-2,224557716
19	TGEV/PRCV	1,42725	1,134	0,29325	20,546505518
20	TGEV	1,42725	1,497	-0,06975	-4,887020494
20	TGEV/PRCV	1,42725	1,327	0,10025	7,023997197
21	TGEV	1,42725	1,514	-0,08675	-6,078122263
21	TGEV/PRCV	1,42725	1,384	0,04325	3,030303030
22	TGEV	1,42725	1,435	-0,00775	-0,543002277
22	TGEV/PRCV	1,42725	1,182	0,24525	17,183394640
24	TGEV	1,42725	1,415	0,01225	0,858293922
24	TGEV/PRCV	1,42725	1,235	0,19225	13,469959713
25	TGEV	1,42725	1,41	0,01725	1,208617972
25	TGEV/PRCV	1,42725	1,296	0,13125	9,196006306
26	TGEV	1,42725	1,427	0,00025	0,017516202
26	TGEV/PRCV	1,42725	1,327	0,10025	7,023997197
27	TGEV	1,42725	1,46	-0,03275	-2,294622526
27	TGEV/PRCV	1,42725	1,417	0,01025	0,718164302
28	TGEV	1,42725	1,47	-0,04275	-2,995270625
28	TGEV/PRCV	1,42725	1,4	0,02725	1,909266071
29	TGEV	1,42725	1,438	-0,01075	-0,753196707
29	TGEV/PRCV	1,42725	1,353	0,07425	5,202312139
30	TGEV	1,42725	1,454	-0,02675	-1,874233666
30	TGEV/PRCV	1,42725	1,318	0,10925	7,654580487
31	TGEV	1,42725	1,439	-0,01175	-0,823261517
31	TGEV/PRCV	1,42725	1,315	0,11225	7,864774917
32	TGEV	1,42725	1,418	0,00925	0,648099492
32	TGEV/PRCV	1,42725	1,317	0,11025	7,724645297
33	TGEV	1,42725	1,4	0,02725	1,909266071
33	TGEV/PRCV	1,42725	1,082	0,34525	24,189875635

35	TGEV	1,42725	1,457	-0,02975	-2,084428096
35	TGEV/PRCV	1,42725	1,334	0,09325	6,533543528
36	TGEV	1,42725	1,444	-0,01675	-1,173585567
36	TGEV/PRCV	1,42725	1,352	0,07525	5,272376949
37	TGEV	1,42725	1,462	-0,03475	-2,434752146
37	TGEV/PRCV	1,42725	1,42	0,00725	0,507969872
38	TGEV	1,42725	1,506	-0,07875	-5,517603783
38	TGEV/PRCV	1,42725	1,38	0,04725	3,310562270
39	TGEV	1,42725	1,564	-0,13675	-9,581362761
39	TGEV/PRCV	1,42725	1,42	0,00725	0,507969872
40	TGEV	1,42725	1,427	0,00025	0,017516202
40	TGEV/PRCV	1,42725	1,289	0,13825	9,686459975
41	TGEV	1,42725	1,426	0,00125	0,087581012
41	TGEV/PRCV	1,42725	1,242	0,18525	12,979506043
42	TGEV	1,42725	1,436	-0,00875	-0,613067087
42	TGEV/PRCV	1,42725	1,359	0,06825	4,781923279
43	TGEV	1,42725	1,388	0,03925	2,750043791
43	TGEV/PRCV	1,42725	1,386	0,04125	2,890173410
46	TGEV	1,42725	1,401	0,02625	1,839201261
46	TGEV/PRCV	1,42725	1,296	0,13125	9,196006306
47	TGEV	1,42725	1,426	0,00125	0,087581012
47	TGEV/PRCV	1,42725	1,225	0,20225	14,170607812
48	TGEV	1,42725	1,469	-0,04175	-2,925205815
48	TGEV/PRCV	1,42725	1,32	0,10725	7,514450867
49	TGEV	1,42725	1,48	-0,05275	-3,695918725
49	TGEV/PRCV	1,42725	1,354	0,07325	5,132247329
51	TGEV	1,42725	1,365	0,06225	4,361534419
51	TGEV/PRCV	1,42725	1,285	0,14225	9,966719215
52	TGEV	1,42725	1,366	0,06125	4,291469609
52	TGEV/PRCV	1,42725	1,318	0,10925	7,654580487
54	TGEV	1,42725	1,374	0,05325	3,730951130
54	TGEV/PRCV	1,42725	1,376	0,05125	3,590821510
53	TGEV	1,42725	1,493	-0,06575	-4,606761254

53	TGEV/PRCV	1,42725	1,313	0,11425	8,004904537
56	TGEV	1,42725	1,395	0,03225	2,259590121
56	TGEV/PRCV	1,42725	1,348	0,07925	5,552636188
57	TGEV	1,42725	1,4	0,02725	1,909266071
57	TGEV/PRCV	1,42725	1,304	0,12325	8,635487826
58	TGEV	1,42725	1,349	0,07825	5,482571379
58	TGEV/PRCV	1,42725	1,228	0,19925	13,960413382
59	TGEV	1,42725	1,4	0,02725	1,909266071
59	TGEV/PRCV	1,42725	1,237	0,19025	13,329830093
none	TGEV	1,42725	1,283	0,14425	10,106848835
none	TGEV/PRCV	1,42725	1,141	0,28625	20,056051848