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Nasal carriage of Methicillin-Resistant *Staphylococcus aureus* among sympatric Free-ranging Domestic Pigs and wild *Chlorocebus pygerythrus* in a rural African setting

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ABSTRACT

Background

Methicillin Resistant *Staphylococcus aureus* (MRSA) nasal carriage in domestic pigs and vervet monkeys is a risk factor for subsequent severe infections in domestic pigs and for dissemination to the human population. This study assessed nasal carriage of MRSA in domestic pigs and sympatric vervet monkeys in a rural African village during an outbreak of a virus hemorrhagic fever suspected to be contracted from wild primates.

This study was conducted during the 2012 Ebola outbreak to determine nasal carriage of MRSA in free-ranging domestic pigs and sympatric freely roaming vervet monkeys using conventional methods. *Staphylococcus aureus* (*S. aureus*) isolated from the anterior nares were tested for susceptibility to commonly used antibiotics and conventional PCR was used to confirm methicillin resistance.

Results

MRSA was significantly more in wild vervet monkeys compared to free ranging domestic pigs ($p=0.003875$). Overall, there was a high level of resistance to tetracycline [90% (63/70) in pigs and 67% (10/15) in vervet monkeys], trimethoprim/sulphamethoxazole [90% (63/70) in pigs and 67% (10/15) in vervet monkeys], and penicillin [83% (58/70) in pigs and 67% (10/15) in vervet monkeys].

Conclusion

The nasal carriage of drug resistant *S. aureus* in freely roaming domestic and wild animals presents a risk for widespread environmental spread of antimicrobial resistance thus presenting a risk for treatment failure in domestic animals, wild animals, and humans.

Introduction

Methicillin-resistant *S. aureus* (MRSA) infections are a global concern with a considerable effect on treatment outcomes and costs. (1) The methicillin resistance gene (*mecA* gene) located in the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) is responsible for the synthesis of an altered penicillin binding protein (PBP) 2a which in turn confers cross-resistance to beta-lactam antibiotics. (2) Animals play a role in the epidemiology of MRSA infection and colonization in humans. (3) The Livestock Associated MRSA (CC 398) has been documented to colonize pigs and less commonly poultry and cattle and it commonly affects veterinarians and farmers in areas with huge pig populations through direct animal contact, environmental contamination, or meat consumption (4).

Given the high extent of low host-specificity among many of the MRSA clonal lineages, it may not always be beneficial to categorize methicillin resistant *Staphylococcus aureus* into Hospital Acquired, Community Acquired and Livestock associated; (5) especially in the developing countries where the animal- human interface is fluid.

In Africa, PVL-positive MRSA (ST5) was described in nasal samples of pigs from Senegal (6, 7) and MRSA ST88 from pigs and humans in Nigeria showed high genetic similarity. (8)

In Uganda, MRSA has been observed in foods of animal origin such as milk (9) and was particularly highly prevalent in domesticated pigs. (10) Interestingly, it was also observed to be highly prevalent in the surgical units of Uganda's national referral hospital. (11)

In the wild, there is no use of antibiotics in most non-captive wild animals especially among those that are categorized as vermin by the Uganda Wildlife Authority (UWA); the UWA defines "vermin" as problem animals that cause damage to people, farms and other assets.(12) Notably, during the dry season when vervet monkey food is relatively scarce in the wild, they migrate closer to homesteads in search of food from gardens. In doing so, they often come close to the free ranging domestic pigs and could potentially transmit zoonotic pathogens to humans.(13) Given their ecological role, non-human primates like the vervet monkeys living at the human-animal interface could potentially play a positive role in the re-establishment of susceptible bacteria after removal of drug pressure (14) However, a previous

study showed a high prevalence of MRSA in both colony-born and wild vervet monkeys(15) and there is need to determine the occurrence of key antimicrobial resistance phenotypes in wild animals in close interface with rural communities in Africa. We are not aware of any study that documented nasal carriage of MRSA among wild vervet monkeys and sympatric free range domestic pigs in a rural African setting. This study investigated nasal carriage of MRSA among free ranging domestic pigs and sympatric wild vervet monkeys in a rural African village.

Results

Staphylococcus aureus was isolated from all the pigs (70/70) and free ranging vervet monkeys (15/15). Of the isolates from pigs, 11.4% (8/70) were resistant to ceftiofur and oxacillin while only , 26.7% (4/15) of the isolates from monkeys were resistant to ceftiofur and oxacillin. The isolates that showed phenotypic resistance to oxacillin and ceftiofur and were confirmed to be Methicillin resistant using the MecA PCR (**Figure 1**). All the isolates that showed phenotypic resistance to oxacillin and ceftiofur were positive for the Mec A gene. MRSA was significantly more in wild vervet monkeys compared to free ranging domestic pigs ($p=0.003875$).

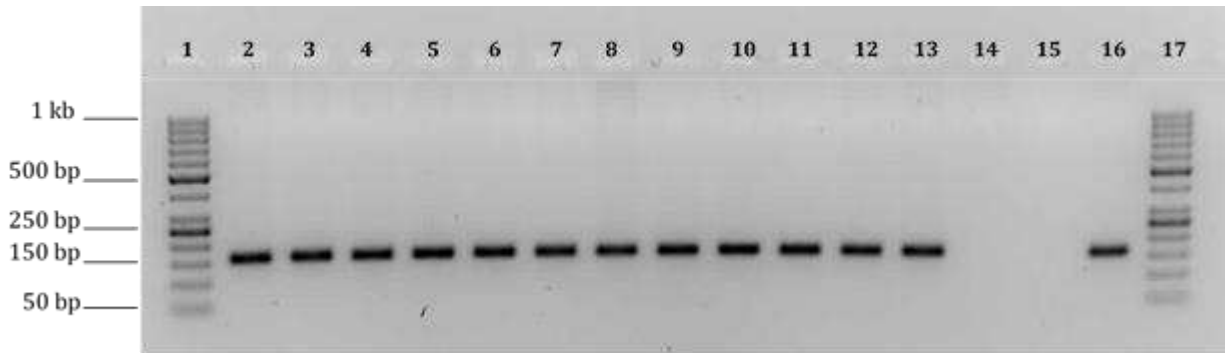


Figure 1 Gel image of the Mec A PCR on suspected MRSA isolates from vervet monkeys and free -ranging domestic pigs in Wakiso district, Uganda

Lanes 1 and 17 represent the 1kb ladder while Lanes 14 and 15 are for the negative controls. Lane 16 shows the positive control. Lanes 2 – 13 are test isolates.

Overall, there was a high level of resistance to tetracycline [90% (63/70) in pigs, 67% (10/15) in vervet monkeys], trimethoprim/sulphamethoxazole [90% (63/70) in pigs, 67% (10/15) in vervet monkeys], and penicillin [83% (58/70) in pigs, 67% (10/15) in vervet monkeys](**Table 1**).

Table 1 Percentage resistant for isolates from domestic pigs and free ranging vervet monkeys

Antibiotic (μg)	Domestic pigs (%R, n=70)	Vervet Monkeys (%R, n=15)
Penicillin G (10U)	83%, 58/70	67%, 10/15
Oxacillin (1 μg)	11.4%, 8/70	26.7%, 4/15
Cefoxitin (30 μg)	11.4%, 8/70	26.7%, 4/15
Erythromycin (15 μg)	1.4%, 1/70	-
Clindamycin (2 μg)	1.4%, 1/70	-
Gentamicin (10 μg)	2%, 1/70	-
Trimethoprim/ Sulfamethoxazole (25 μg)	90%, 63/70	67%, 10/15
Tetracycline (30 μg)	90%, 63/70	67%, 10/15
Chloramphenicol (30 μg)	1.4%, 1/70	-
Ciprofloxacin (5 μg)	1.4%, 1/70	-
Fusidic acid (10 μg)	-	-
Rifampicin (30 μg)	2.9%, 2/70	6.7%, 1/15
Linezolid (30 μg)	2.9%, 2/70	-
Vancomycin	-	-
Tigecycline (15 μg)	-	-

Discussion

Key findings of the study

MRSA was significantly more in free ranging vervet monkeys compared to free ranging domestic pigs ($p=0.003875$). A previous study showed a 29.7% prevalence of MRSA among domestic pigs in the South Western region of Uganda.(10) This is higher than the 11.4% prevalence reported in this study. Besides being done in a different part of Uganda, the difference in resistance rates could be explained by the difference in pig husbandry methods used – this study focused on pigs that are free-ranging. The tendency to use antibiotics is higher for intensively raised pigs as opposed to the free ranging pigs. The higher prevalence of MRSA in wild vervet monkeys compared to free-ranging domestic pigs could suggest that wild vervet monkeys are more exposed to antibiotics than free-ranging pigs. Notably, the destruction of the natural ranges chiefly for agriculture and human settlement has impacted the level of interaction between vervets and humans as well as with domestic animals. Additionally, the existence of vervets in colonies of 10 to 50 increases the likelihood of spread of different antibiotic resistance phenotypes to those in the same colony.

Overall, there was a high level of resistance to tetracycline [90% (63/70) in pigs, 67% (10/15) in vervet monkeys)], trimethoprim/sulphamethoxazole [(90% (63/70) in pigs, 67% (10/15) in vervet monkeys)], and penicillin [(83% (58/70) in pigs, 67% (10/15) in vervet monkeys)].

The high level of resistance to commonly used antibiotics by *S. aureus* isolated from pigs is reminiscent of findings of an earlier study; (10) and of several others done in Uganda on *S. aureus* from human and non-human sources that reported high resistance rates to tetracyclines, penicillin, and trimethoprim/sulphamethoxazole. (16-19)

Recommendations

This study was conducted in the dry season only and did not carry out spa typing to further characterize the isolates. Further research needs to consider more free ranging wildlife other

than just vervet monkeys and should be conducted over both the wet and dry seasons, with the resultant isolates further characterized by spa typing to determine the livestock associated spa types in humans and wild animals.

Conclusions

Control strategies for antimicrobial resistance at the human-animal interface in Luwero and Wakiso need to consider the role of free ranging pigs and vervet monkeys in the epidemiology of key resistance phenotypes such as MRSA. This study demonstrates the fluidity of the interface between animals in the wild such as the vervets and the human communities. MRSA has spread beyond the human communities and into the nearby wildlife communities and should therefore be considered to be widespread in the environment. Efforts to control antimicrobial resistance in these regions must factor in the nearby wildlife communities.

Research Methods and design

Luwero district (**Figure 2**) is in the central part of Uganda and has recently suffered two Ebola outbreaks thought to be linked to non-human primates. Kakute village is located near a local river called Lwajali which is a tributary of the Sezibwa river which drains from L. Kyoga to the North and drains downstream south to the Lake Victoria. It starts from the swamp, west of the village of Kisweera, in Mukono District, the Central Region of Uganda and It flows north to empty into River Sezibwa in Kayunga District at its border with Luweero District, east of the village of Kiziba. The region around the Lwajali river in Kakute village is lush green and habited by various forms of wildlife (and humans) including vervet monkeys. During the ecological surveillance studies to determine the non-human source of Ebola virus responsible for the 2012 Ebola outbreak, simultaneously, nasal swabs were collected to detect MRSA from free ranging pigs (Since the Reston Ebola virus was shown to affect pigs, domestic pigs were also screened as possible sources of the Ebola virus and nasal swabs were collected and tested for MRSA) and wild vervet monkeys. The domestic pigs in this region were different crossbreeds of the large white and large black, were free ranging and kept in small numbers at a subsistence level as previously described. (20) The vervets in this area were identified by ecologists from the Uganda Wildlife Authority (UWA) as *Chlorocebus pygerythrus* and are commonly encountered in this area destroying peoples' gardens and are therefore categorized by UWA as 'vermin'.

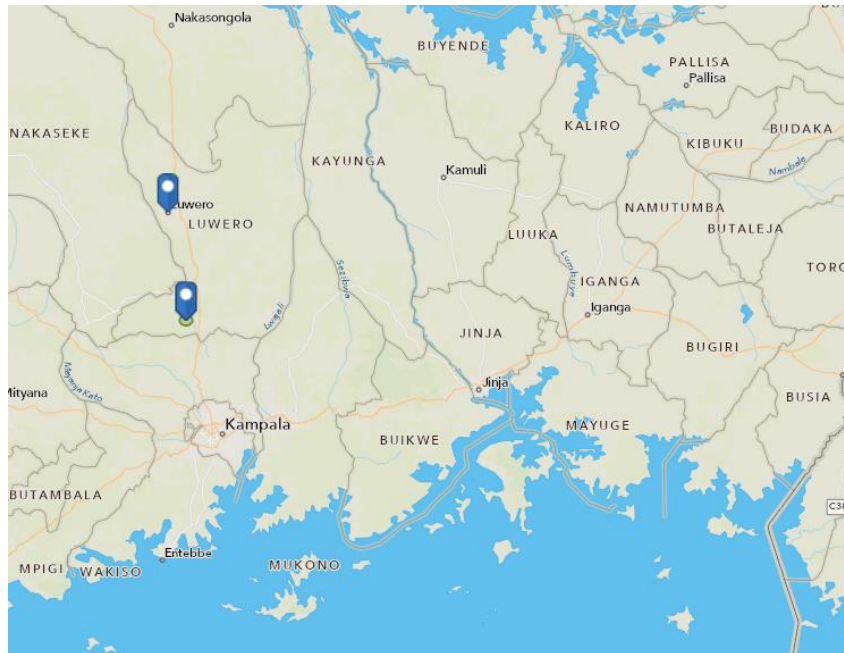


Figure 2 Study Area in Luwero district, Kakute village, Uganda.

The blue pointer with a green base indicates the location of kakute village in Luwero district, Uganda

Sample collection

Free ranging domestic pigs: We collected nasal swabs from free ranging pigs in all homesteads that kept them. The pigs were restrained using a pig strainer prior to sample collection. Ultimately, forty homesteads in Kakute village (GPS co-ordinates 0.60075, 32.5012), Luwero district were selected and visited between February 2012 and September 2013. Free-ranging pigs were restrained, and the anterior nares swabbed using sterile cotton swabs (BBL CultureSwab plus Amies Medium, Becton Dickinson, New Jersey, US). The nasal swabs were immediately transported to the veterinary microbiology laboratory at Makerere University and processed within 3h of collection. During the same period, a team of wildlife professionals (from the Uganda Wildlife Authority) visited the nearby forests, swamps or gardens to catch wild vervet monkeys and swab their anterior nares. An air-powered darting rifle and syringe darts loaded with a mixture of Ketamine-Rompun were applied during the capture process which was done in accordance to the animal welfare regulations. The swabs were labeled with the animal species, date of collection, and sample type. The swabs were temporarily held at

4°C and processed within 3h of collection at the veterinary microbiology laboratory at Makerere University.

Isolation of *S. aureus*

In total, 85 nasal swabs were collected from free-ranging domestic pigs (70 swabs) and wild vervet monkeys (15 swabs). The swabs were inoculated onto Mannitol Salt Agar (MSA) (Veterinary Microbiology laboratory, Makerere University), and incubated at 37°C in ambient air. After 24 hours of incubation, growth characteristics and colony morphology were evaluated. Subsequently, for each plate, mannitol fermenting colonies were selected for further identification. Among the morphologically similar mannitol fermenting colonies, only one characteristic colony was selected for further processing while morphologically distinct colonies were treated uniquely. Additionally, the non-mannitol fermenting colonies suspected to be *S. aureus* were subjected to further identification.

Deoxyribonuclease (DNase) and tube coagulase test

Suspected colonies of *S. aureus* were inoculated onto DNase agar (Difco Laboratories, Detroit, Mich.), and incubated at 37°C for 24 hours following the manufacturer's instructions. A DNase positive result was identified by a strong clear zone around the bacterial streak as previously described.(21) The formation of a firm clot on the tube coagulase test was indicative of *S. aureus* as previously described.(22)

Suspect *S. aureus* isolates that were positive on the DNase and tube coagulase test were confirmed using the API staph Ident. system (bioMérieux SA, Marcy l'Etoile, France) following the manufacturer's instructions as previously described.(23)

Antimicrobial Susceptibility Testing

To resuscitate the *S. aureus* isolates stored in Brain Heart Infusion (BHI)/glycerol solution were sub-cultured onto Columbia blood agar BA (2% agar and 5% sheep blood) and incubated in ambient air at 37°C overnight. The susceptibility testing was performed using the Kirby-Bauer disc diffusion method and interpreted according to the Clinical Laboratory Standard Institute guidelines. The antibiotic discs included penicillin G (10U), oxacillin (1 µg), cefoxitin (30 µg), erythromycin (15 µg), clindamycin (2 µg), gentamicin (10µg),

trimethoprim/sulphamethoxazole (25µg), tetracycline (30µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), fusidic acid (10 µg), rifampicin (30 µg), linezolid (30 µg), and tigecycline (15 µg) (Mast group Ltd., Merseyside, UK).

Interpretative zone diameter for tigecycline (15 µg), fusidic acid (10µg) not included in CLSI guidelines, were interpreted using the European Committee on Antibiotic Susceptibility Testing (EUCAST) guidelines. *S. aureus* ATCC 25923 and BAA-976 were used as quality control strains for methicillin-susceptible and resistant strains respectively and were included in each experiment. Isolates showing resistance to oxacillin and ceftiofur were further confirmed by PCR targeting the *mecA* gene. Susceptibility testing to vancomycin was performed for MRSA strains using the VITEK2 system (BioMerieux, Marcy l'Etoile, France) at the veterinary microbiology laboratory, Makerere University. To identify inducible macrolide-lincosamide-streptogramin B (MLS_B) resistance phenotype, the D-test was performed according to the CLSI guidelines. A “D-shaped” inhibition zone confirmed inducible resistance to clindamycin (2µg) using erythromycin (15µg).(24) The ATCC strain BAA-977 was used as a quality control strain for ICR and was included with each experiment.

Nucleic Acid extraction

The stored *S. aureus* isolates were sub-cultured onto Columbia based blood agar BA (2% agar and 5% sheep blood) and incubated in ambient air at 37°C overnight. DNA was extracted using the heat-lysis protocol (25) with a slight modification. A loopful from an 18–24-hour old culture on BA was suspended in 200µl of the Tris buffer, heated at 95°C for 15 minutes and then centrifuged at 10,000g for 5 minutes. A volume of 50µl of the supernatant was kept at -20°C for molecular characterisation.

Confirmation of resistance to ceftiofur and oxacillin by *mecA* PCR

S. aureus isolates that showed resistance to oxacillin were screened for the *mecA* gene using end-point PCR as described previously.(26)

The PCR reactions contained a final concentration of 1X Super-Therm buffer, 1.5 mM MgCl₂, 1.5U Super-Therm *Taq* polymerase (JMR holdings, London, UK), and 0.2 mM deoxynucleotide

triphosphate mix (dNTPs) (Thermo Scientific, Wilmington, USA), and a primer concentration of 0.25µM for each primer (**Table 2**).

Table 2 Primers used to amplify the mecA gene

Primers	5' – 3' primer sequence	Amplicon size
MecA forward primer	TCCAGATTACA ACTTCACCAGG	162bp
MecA reverse primer	CCACTTCATATCTTGTAACG	

A volume of 5µl DNA template was added to the mixture to make a total volume of 50µl. The PCR amplification was performed in the Applied Biosystems 2720 Thermal cycler (Applied Biosystems, Carlsbad, USA). The thermal cycling temperatures were as follows: denaturation at 94°C for 4 minutes, followed by 30 amplification cycles of 94°C, 53°C and 72°C for 30 seconds each and final elongation at 72°C for 3 minutes. The positive control strain and PCR grade water as the non-template control were used in each PCR run. The PCR products were loaded on 2% agarose gel in 1% Tris-acetic acid-EDTA buffer at 80V for 1.5h and visualized with ethidium bromide.

List of abbreviations

U.W.A = Uganda Wildlife Authority

CoVAB = College of Veterinary Medicine Animal Resources and Biosecurity

Figure Legends

Figure 1 Gel image of the MecA PCR on suspected MRSA isolates from vervet monkeys and free-ranging domestic pigs in Wakiso district, Uganda

Figure 2 Study Area in Luwero district, Kakute village, Uganda.

The blue pointer with a green circle indicates the location of kakute village in Luwero district, Uganda

Declarations

Ethics approval and consent to participate

Ethical approval for the study was received from the research and ethics committee of the College of Veterinary Medicine Animal resources and Biosecurity of Makerere University

(HREC 2012/034). Written informed consent in the local language detailing the purpose of the study, eligibility for participation, expected duration of participation, description of the procedure, and any possible discomfort and risks to the animals was received from the owners of the free ranging domestic pigs prior to sample collection.

All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not Applicable.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

We do not have any competing interest to declare

Funding

This study was funded by the Ministry of Agriculture Animal Industry (MAAIF) of Uganda and Fisheries and the Uganda Wildlife Authority (UWA).

Authors' contributions

Dr John Bosco Kalule planned the study and participated in sample collection, processing, and writing of the manuscript. Mr Ssendawula Simon Peter participated in the sample processing and in the writing of the manuscript. Ms Nakintu Zalwango Valeria participated in the sample processing and in the writing of the manuscript.

Acknowledgements

Not Applicable

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Figures

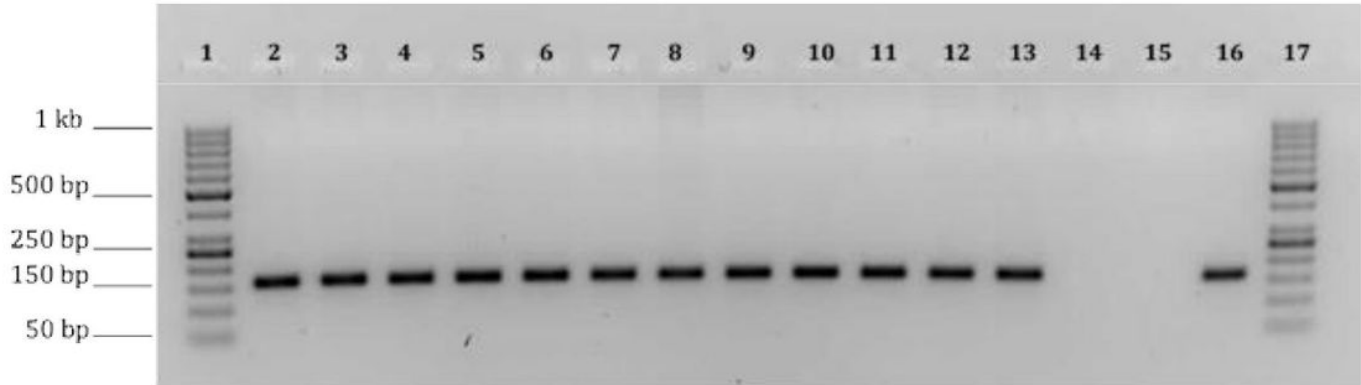


Figure 1

Gel image of the Mec A PCR on suspected MRSA isolates from vervet monkeys and free -ranging domestic pigs in Wakiso district, Uganda Lanes 1 and 17 represent the 1kb ladder while Lanes 14 and 15 are for the negative controls. Lane 16 shows the positive control. Lanes 2 – 13 are test isolates.

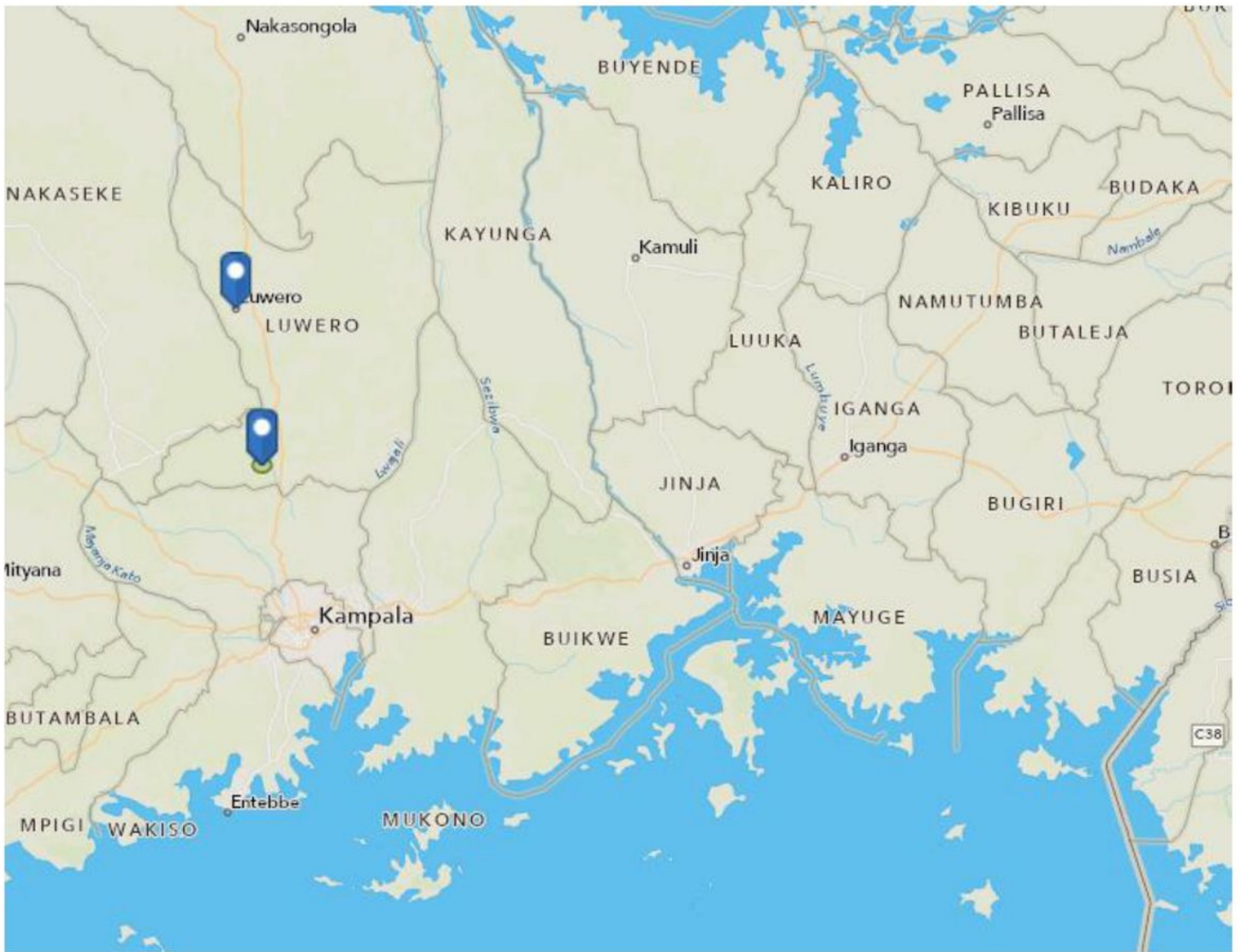


Figure 2

Study Area in Luwero district, Kakute village, Uganda. The blue pointer with a green base indicates the location of kakute village in Luwero district, Uganda Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.