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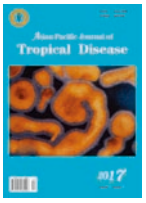

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Occurrence of Norovirus in pig faecal samples in the Eastern Cape, South Africa

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ABSTRACT

Objective: To determine the occurrence of Norovirus infection amongst pigs in selected communities in the Eastern Cape, South Africa.
Methods: Stool samples were collected from pigs in two commercial farms using sterile swab sticks between September and November 2015. The samples were analysed by RT-PCRs and nested PCRs utilizing the p289/p290 primers pair targeting RNA-dependent RNA polymerase section of Norovirus as previously described in literatures. All positive isolates were sequenced, edited and analysed phylogenetically making use of Geneious version 9.1.5 software.
Results: Out of the one hundred and twenty faecal samples screened, four samples (3.3%) from farm A and one sample (0.8%) from farm B were positive for Norovirus. Phylogenetic analysis showed that out of the all the positive isolates analysed, three nucleotide sequences (FH14, FH20 and TS05) clustered with swine enteric Caliciviruses, while FHO5 clustered with human Norovirus GII and FH13 clustered closely with bat Caliciviruses.
Conclusions: Results obtained in this study revealed that swine do harbour Norovirus and could be a source for human infection under compromised sanitary conditions.

1. Introduction

Caliciviruses are small non-enveloped single stranded positive sense RNA viruses, which have a genomic size of 7.4–8.3 kb[1,2]. Norovirus and Sapovirus are pathogenic species of Caliciviruses identified in animals and in humans and are known to cause widespread foodborne diseases[3,4]. Norovirus genome has three open reading frames (ORFs) where ORF1 encrypts a polyprotein with six non-structural proteins, ORF2 and ORF3 codes for structural proteins called main capsid protein VP1 and minor protein VP2[5,6] and it is categorized into five genogroups (GI–GV)[7,8]. Pigs are susceptible to human Norovirus genogroup II, which is the main predominant genogroup causing gastroenteritis in human beings[9]. Pig strains of Norovirus are hereditarily similar to human strains which were known to replicate and show a mild pathogenicity in gnotobiotic pigs under experimentally conditions[10,11].

Norovirus is mostly detected in finisher swine, whereas Sapovirus has been found in all different age groups of swine[10,12]. Swine illnesses are associated with porcine Sapovirus while the influence of Norovirus contagions in swine is unknown[3]. Some strains of Caliciviruses (Norovirus and Sapovirus) have the potential for zoonotic transmission since they are hereditarily related to humans[13] and studies have shown that some domestic pigs serve as the reservoir of these viruses[14].

Human Caliciviruses (Norovirus and Sapovirus) are highly incriminated in gastroenteritis[15-17]. However, the invasive role of Norovirus and its effect in animals is vague. Numerous epidemiological studies conducted globally identified Norovirus

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All experimental procedures involving animals were conducted in accordance to ethics of handling experimental animals and approved by University of Fort hare research ethics committee.

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in pigs without symptoms[18,19]. In Brazil, 58% of faecal samples collected from adult animals of asymptomatic pigs tested positive for Norovirus[20].

Norovirus have been observed amongst pigs from high income countries, suggesting the significance to monitor the incidence of Norovirus in swine with a view to assessing the risk in humans since Norovirus is highly diverse and new strains are continually evolving and having also the possibility of interspecies spread[10]. The genetic relatedness of Norovirus and Sapovirus strains can be identified by sequence analysis which allows cluster and strain alignments thus facilitating the study of Norovirus and Sapovirus distribution and spread patterns within a population[21].

The prevalence of Norovirus as the cause of outbreak in humans has been reported in South Africa but studies on the distribution of Norovirus in swine are rare. South Africa has the largest population of pigs in Southern Africa where pigs are reared for commercial purpose[22]. The pig industry in South Africa is comparatively huge within the overall South African agricultural sector which comprises commercial intensive sector and an extensive sector[23]. Furthermore, to determine the incidence of Norovirus infection in pig-producing provinces of South Africa is essential, since there is a probability that Norovirus could affect the medical status and productivity of pig herds. There is paucity of information on the prevalence of Norovirus among swine in the Eastern Cape of South Africa, which motivated this current research.

2. Materials and methods

2.1. Ethical statement

The ethical clearance of this research was acquired from the ethics committee of the University of Fort Hare while approval to carry out the study was sought from the Department of Agriculture, Forestry and Fisheries in Eastern Cape Province, South Africa. The permission to collect samples was granted by the farm owners of the farms prior to sample collection.

2.2. Study area

The research was carried out in two different farms (farm A and farm B) situated in Eastern Cape. Eastern Cape is the second biggest region in South Africa. It has a population of 6.7 million and is made up of eight district municipalities. Farm A is situated in Alice, South East of the University of Fort Hare along R63 route linking Alice to King Williams Town. It is situated in Nkonkobe Local Municipality under Amatole District Municipality. Farm B is situated in Tsolo 2 km North East away from town and it is located in Mhlontlo Local Municipality within OR Tambo District Municipality. Figure 1 shows the map of district municipalities in Eastern Cape where the two selected farms are located.

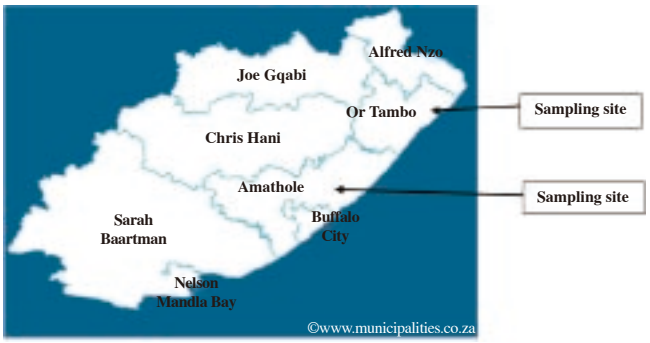

Figure 1. Eastern Cape map showing district municipalities selected for sampling.

2.3. Sample collection

A standard biosafety protocol for maintaining required personal hygiene was followed before entering the farms and collecting the samples. Subsequently, 120 stool samples were collected using sterile swab sticks from pigs (weaners and growers) between September and November 2015, of which 100 samples were collected from farm A and 20 from farm B comprising piglets ranging between the ages of 3 to 90 days. Samples were transported on ice to the Applied and Environmental Microbiology Research Group laboratory at the University of Fort Hare for analysis. The specimens were suspended in 1% of phosphate buffered saline, vortexed, centrifuged at 5 000 r/min for 5 min and the supernatant was collected and stored at –80 ℃ until use.

2.4. Extraction of RNA and RT-PCR

RNA was extracted from 160 μL of 10% (w/v) faecal suspensions using Quick-RNA extraction kit (Zymo Research) following the manufacturer's instructions in the laboratory of the Applied and Environmental Microbiology Research Group, University of Fort Hare, Alice, South Africa. The viral RNA was stored at –80 ℃ until use. The cDNA was generated in a one-step RT-PCR in a 25 μL reaction mixture comprising 0.25 μL reverse transcriptase (superscript III), 12.5 μL of the master mix, 1.5 μL each of the primers (reverse and forward), 4.25 μL of RNase nuclease free water and 5 μL of RNA. The reverse transcriptase assay was performed immediately at 50 ℃ for 1 h.

2.5. Nested PCR

Nested PCR was performed using the cDNA as template to generate the desired amplicons. A universal primer pair p290/p289 targeting the RNA-dependent RNA polymerase (RdRp) region of Norovirus was used for the detection of Norovirus. The sequences of the primers are as follows: forward primer 5'-GATTACTCCAAGTGGGCTCCAC-3' and reverse primer 5'-TGACAATGTAATCATCACCATA-3', which amplified a 320 bp products from Norovirus under the following conditions: initial denaturation at 94 ℃ for 3 min, then 35 cycles of

denaturation at 94 ℃ for 1 min, annealing at 52 ℃ for 1 min and extension at 72 ℃ for 1 min, followed by final extension of 72 ℃ for 1 min[9]. The amplified products were verified in 1.5% agarose gel, stained with ethidium bromide, electrophoresed at 120 V for 45 min using 0.5% TBE buffer and visualized using Alliance 4.7 UV transilluminator.

2.6. Bioinformatics analysis

The amplified amplicons were sequenced and sequence editing was performed using Geneious R9.1.5 version (Biomatters Limited) and the generated nucleotide sequences were subjected to homology search by means of the BLAST 2.0 program in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The edited sequence data were compared with homologous sequences of previously characterized Norovirus in the GenBank. The sequences with high percentage similarity were employed in the phylogenetic analyses of the amplified amplicons. The positive samples (amplified amplicons) were also analyzed for genotypes assignment using Norovirus typing tool (http://www.rivm.nl/mpf/norovirus/typingtool). Phylogenetic tree was drawn using Geneious bioinformatics software version 9.1.5 and bootstrapped with 1 000 replicates.

2.7. GenBank accession number

The generated sequences were submitted to the GenBank and the accession numbers for our sequence were assigned: FH05 (KX907838), FH13 (KX907839), FH14 (KX907840), FH20 (KX907841), and TS05 (KX907842).

3. Results

In this study, out of one hundred and twenty faecal samples that were screened using RT-PCR assay, four samples (3.33%) from farm A and one sample (0.83%) from farm B tested positive. The overall percentage of positive samples was 4.2% which clearly showed low occurrence of Calicivirus infection among the herds studied.

All the positives samples were analysed by blasting using the BLAST tool as implemented in National Center biotechnology information (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The nucleotide sequence of FH05 showed percentage similarity of 82% identity with human Norovirus (KP857570, GU012326 and GU012342) from China and Brazil and swine Sapovirus (GU991614) from Italy. However, with the phylogenetic tree (Figure 2) FH05 was closely related to human Norovirus GII (KP857570) isolated from China. The nucleotide sequences of FH20, FH14 and TSO5 showed 80% similarity to porcine enteric Sapovirus (EF183479, KT895958, and KF241960) from Brazil and China and swine enteric viruses (AB22300 and AB521790) from Japan after blasting. Analysis by phylogenetic method showed they were closely related to swine enteric Caliviruses (AB223000) and porcine Sapovirus (EF183478). The nucleotide sequence of FH13 appeared to be 100% identical to porcine enteric Sapovirus (KF241959) from Brazil; further analysis revealed that on the phylogenetic tree it clustered with bat Caliciviruses (KJ652318). However, all the sequences in this study were not assigned when the Norovirus tool (http://www.rivm.nl/mpf/norovirus/typingtool) was used in order to determine the genotypes and genogroups of the positive isolates.

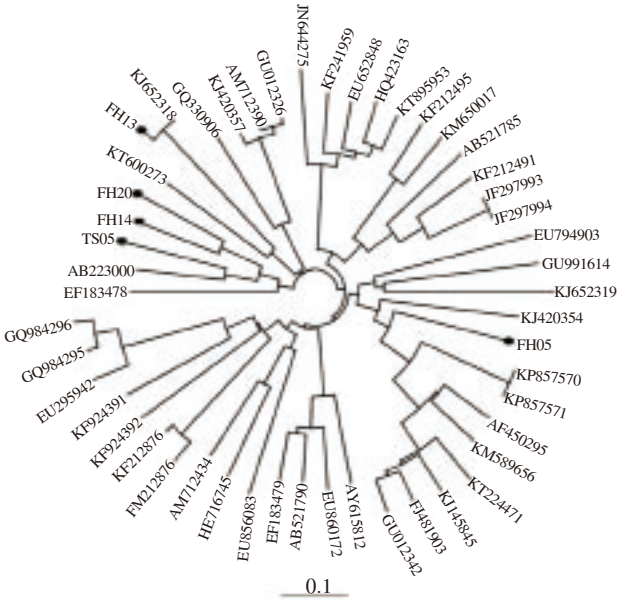

Figure 2. A phylogenetic analysis of Norovirus sequences obtained from faecal samples of swine in the Eastern Cape, South Africa.
Analysis was based on the amplified 300 base-pair of the RdRp region of Calicivirus. Phylogenetic tree was created using Geneious software version 9.1.5 and bootstrapped with 1 000 replicates and accession numbers for the reference strains used in drawing the phylogenetic tree are: AB521785; KF212495; JF297993; JF297994; KF212491; KT895953; HQ423163; EU652848; KF241959; JN644275; KT600273; AB223000; EF183478; AB521790; EU860172; EF183479; AY615812; EU856083; GQ984295; GQ984296; EU295942; GU991614; KF924391; KF924392; KJ652319; GU012342; FJ481903; KM589656; AF450295; KT224471; KJ145845; KP857570; KP857571; GU012326; KJ420357; GQ330906; KJ420354; EU794903; AM712434; FM212876; KM650017; AM712390; HE716751; HE716745; KJ652318.

4. Discussion

Reports on Norovirus prevalence in faecal samples are lacking in Eastern Cape of South Africa and subsequently this is the first study to document occurrence of Norovirus in swine within the region. The results obtained in this study showed low occurrence of Norovirus among pigs (4.2%) in both farms, however these results were high compared to results obtained from other studies in which the occurrence rates detected were 0.5% in Korean pigs[24], 1.2% in asymptomatic swine in Slovenia[25] and 0% in US pigs[26]. The difference in the occurrence rate for detecting Norovirus in swine may be attributed to the geographical sites, ages of swine and variation in detection methods being used[27]. Previous studies have documented many sets of primers that have been utilized to detect the occurrence of porcine Norovirus with comparably minimal identification rate which indicates that Norovirus contagions are

certainly rare amid swine[3]. Furthermore, a study carried out by Scheuer *et al.*[10] documented high occurrence of Norovirus in US finisher pigs (18.9%). Infection rates of porcine enteric Calicivirus can be impacted by hygienic conditions of the farm[9] and a possibility of cross species infection could happen in humans who do not maintain proper hygiene. Also poor sanitation, contaminated water or food can result in human illnesses and this can lead to outbreaks. Similarly, countries such as Botswana (31%), Cameroon (29.6%) and Burkina Faso have documented high occurrence of Norovirus infections in children devoid of symptoms[28-30]. A survey carried out by Mans *et al.*[31] in South Africa documented that 42.8% of children with Norovirus were immunocompromised.

With regard to phylogenetic analysis, isolate FH05 clustered with Norovirus GII isolated from human faecal sample in a study conducted by Li *et al.*[32]. Research has revealed that human GII Norovirus strains (GII.2, 3, 4 and 13) have been infrequently identified from swine in Asia[9,12]. Human and porcine Norovirus sequences within GII are closely related and this shows that animals can possibly be reservoirs for human strains[33]. A possibility of co-infection could occur in humans who do not take central precautions and this is a risk factor for outbreaks. Studies have reported high occurrence rate of antibodies against Norovirus GII in pigs thus suggesting that swine could possibly be infected by human Norovirus GII[26]. Another study conducted in USA reported that human GII.4 variant and GII.2 were capable of replicating and inducing an immune reaction in gnotobiotic swine[24]. Subclinical cases of human Norovirus infections have been observed in pigs thus suggesting that viruses from humans could thrive in the host increasing the chances of a recombination with strain from pigs thereby leading to an evolution of a new variant with an enhanced pathogenicity, replicative capacity and epidemiological implications[14,34,35].

In this study, further analysis also revealed that sequences (FH20 and FH14) obtained from farm A were found to be closely related to sequence TS05 obtained from farm B separated by a distance of about 340 km. This indicates that there might be a possibility that they are from the same breed or farm A supplied pigs to farm B as farm A is a breeder farm. There could also be an unexpected coincidence of the presence of the strains in both farms. The phylogenetic tree showed they were closely related to swine enteric Caliciviruses. Short phylogenetic diversity can be detected within these species with few branches, thus showing a short term evolutionary diversification. Furthermore, sequence FH13 clustered with bat calicivirus, which shows that a cross-infection between bat and swine may have occurred between these two species. All the nucleotide sequences in this study showed a greater similarity to RdRp region of Caliciviruses after blasting on the NCBI page but on the Norovirus tool they were unassigned due to the relatively short sequence region of RdRp that was analysed.

Although there is low detection rate of Norovirus in this study, the cumulative discovery of Norovirus as cause of illness and the limited success in stopping outbreaks of disease have led to the consideration of vaccines as a potential means for the control of the illness[36]. Presently, no vaccine is available for Norovirus illness as majority of Norovirus vaccines are still at different phases of development and in clinical trials. Furthermore, there are no antiviral drugs for the treatment of Norovirus infection. Therefore, it is important to adopt adequate hygienic measures in order to control infections and spread of the virus. Adequate sanitary conditions and quarantine of sick pigs are necessary measures to prevent illness and outbreaks.

These results reveal low occurrence of Norovirus among studied piggery farms in Nkonkobe and Mhlontlo municipalities, Eastern Cape. Even though there is low occurrence of Norovirus among the pigs sampled, this may pose some level of health risk to farm workers and possible transmission to pork consumers if the carcasses are not handled with precautionary measures as human contagions have been documented in different regions globally after consumption of improperly cooked pork and foods. A wider epidemiological implication could arise if infected faecal materials are washed into bodies of water through storm drains thus resulting in an outbreak. The limitation of this study is that only two sites and few samples were analysed. Further studies involving multiple sites and larger number of samples are required for analysis to detect the occurrence of this virus in the Eastern Cape of South Africa.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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