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Emergence of norovirus GI.2 outbreaks in military camps in Singapore

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SUMMARY

Background: Simultaneous acute gastroenteritis (AGE) outbreaks occurred at two military camps. This study details the epidemiological findings, explores possible origins, and discusses preventive measures.
Methods: Investigations included attack rate surveys, symptom surveys, hygiene inspections, and the testing of water, food, and stool samples. DNA/RNA was extracted from stool samples and amplified via real-time reverse transcription PCR (RT-PCR). Partial and full-length capsid nucleotide sequences were obtained, phylogenetic relationships inferred, and homology modelling of antigenic sites performed.
Results: The military outbreaks involved 775 persons and were preceded by two AGE outbreaks at restaurants in the local community. The outbreak was longer and larger in the bigger camp (21 days, attack rate 15.0%) than the smaller camp (6 days, attack rate 8.3%). Of 198 stool samples, norovirus GI.2 was detected in 32.5% (larger camp) and 28.6% (smaller camp). These were essentially identical to preceding community outbreaks. Antigenic site homology modelling also showed differences between identified and more common AGE outbreak strains (norovirus GII.4).
Conclusion: Differences observed highlight difficulties in controlling person-to-person outbreaks among large groups in close proximity (e.g., military trainees). Distinct differences in antigenic sites may have contributed to increased immunological susceptibility of the soldiers to infection.

1. Introduction

Norovirus is the most common cause of acute gastroenteritis (AGE) outbreaks globally.[1] A single-stranded positive-sense RNA virus of the *Caliciviridae* family, norovirus is divided into five genogroups, three of which (GI, GII, and GIV) are known to cause disease in humans.[2] Of the more than 25 genotypes, GII.4 is most commonly implicated as the cause of AGE outbreaks.[3]

The resilience and persistence of norovirus in the environment allows for its spread through a wide range of common and unexpected infective sources.[4,5] It also spreads rapidly in close communities, causing high attack rates in nursing homes, childcare centres, and cruise ships. As such, efforts to contain its spread need to be meticulous for success.[6] Norovirus is also a challenge to the military. Unique operational environments, such as an increased population density, result in the easy spread of norovirus within military populations, and large outbreaks have been documented previously across the world.[7–9]

In September 2013, simultaneous AGE outbreaks occurred at two military camps in Singapore, involving a total of 775 persons. This study aimed to determine the epidemiology of the outbreaks, investigate its origins (including the use of phylogenetic studies and structural modelling), and discuss measures to prevent future occurrences.

2. Methods

2.1. Epidemiology

Singapore is a tropical city-state in Southeast Asia, and a global travel and trade hub with large numbers of travellers entering the

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city daily. Singapore has a conscript military system – all males who finish high school or equivalent are enlisted for 2 years. Soldiers typically reside in military camps during the working week and return home on weekends if there are no military activities. This results in constant interactions between military personnel and the general community in Singapore.

Primary healthcare for military personnel is provided by clinics within the military camps, which also serve as on-site surveillance for infectious diseases. In the Singapore military, AGE is defined as the rapid onset of two or more episodes of vomiting and/or three or more episodes of diarrhoea. A cluster of 10 or more cases meeting these criteria across 24 h (linked either by space or a common food source) constitutes an outbreak. It is mandatory for doctors to inform the military system's epidemiology department when an outbreak is suspected.

On September 24, two military camps reported suspected AGE outbreaks. Epidemiology teams were sent to conduct investigations and implement control measures. Routine outbreak investigations included attack rate surveys for food consumed 2 days prior to the first symptoms, symptom surveys, hygiene inspections, and the testing of water, food, and stool samples. Water samples were obtained from common water sources at food halls and accommodation areas of the affected soldiers. Food samples, if available, were obtained from the food hall (these were stored routinely in freezers for 2 days immediately after cooking for future testing if required) and sent for culture and testing. Stool samples of cases and food handlers were sent for PCR testing.

The number of soldiers reporting sick with AGE was monitored daily by the camp clinic. Control measures were centred on the escalation of personal and environmental hygiene. These included the separation of affected and unaffected soldiers, enforcement of hand-washing and hygiene, ensuring food and water safety, and disinfection of communal areas with sodium hypochlorite.

We describe herein the epidemiological curve, symptoms experienced by the affected soldiers, and results of food, water, and stool tests.

2.2. Sample preparation, nucleic acid extraction, and diagnostic tests

DNA/RNA extraction from stool samples was performed as described previously by Loh et al.[10] A previous work demonstrated that the same procedure was able to isolate total nucleic acid including viral RNA from stool as well.[11] The assays used for eight bacterial pathogens were a mix of those developed in-house and published assays modified into multiplex PCRs and validated with test panels from various external quality assessment providers. The real-time reverse transcription PCR (RT-PCR) to detect norovirus followed a protocol developed by Jothikumar et al.[12]

2.3. RT-PCR amplification of the full length ORF2 region of norovirus

Reverse transcription was performed by first incubating 8 μl of RNA template with 1 mM of each deoxynucleoside triphosphate (dNTP; dATP, dCTP, dGTP, dTTP) and 5 μM oligo$(dT)_{20}$ primer (Invitrogen) at 65 °C for 5 min in a 10-μl reaction, followed by rapid cooling on ice. The resultant solution was added to a 10-μl reaction mixture containing a final concentration of 1× reverse transcription buffer (Invitrogen), 10 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 2 U RNaseOUT (Invitrogen), and 200 U of SuperScript III reverse transcriptase, and incubated for 50 min at 50 °C. Reverse transcription was terminated by increasing the temperature to 85 °C for 5 min.

The primer set G1SKF (5′-CTG CCC GAA TTY GTA AAT GA-3′′) and CapA (5′- GGC WGT TCC CAC AGG CTT-3′′) was used to amplify the full-length ORF2 region (about 1700 bp) of the norovirus detected in the outbreak samples.[13,14] The PCRs were performed with 3 μl added to 27 μl of a PCR mixture with (final concentrations) 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 100 μM each dNTP, 0.33 μM each primer, and 1.5 U Taq polymerase (Invitrogen). The PCR was carried out at 95 °C for 5 min, followed by 40 cycles of 95 °C, 48 °C, and 72 °C for 30 s, 1 min, and 2 min, respectively, and then a final elongation step at 72 °C for 7 min. Five microlitres of PCR product was electrophoresed on 1% agarose in 0.5× TBE (Tris–borate–EDTA) buffer and visualized by Midori Green staining. The remaining PCR was sent for Sanger sequencing.

2.4. Phylogenetic analysis of capsid sequences

Partial and full-length capsid nucleotide sequences were obtained from (1) community isolates from the National Public Health Laboratory, Ministry of Health, Singapore, (2) closest BLAST hits (99% identity) from Asia and outside Asia,[15] and (3) GII norovirus sequences from a recent study in Singapore from Genbank.[16] CD-HIT was used to cluster reference sequences based on 98% identity and these were aligned to community isolates and outbreak samples in MAFFT,[17] using the E-INS-i algorithm in two steps; aligned full-length sequences were used as a seed alignment for the addition of shorter sequences from community and military outbreaks.[18] Phylogenetic relationships were inferred using the maximum likelihood method based on the Tamura–Nei model.[19] Initial tree(s) for the heuristic search were obtained by applying the neighbour-joining and Bio-NJ methods to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and bootstrapped 1000 times. The analysis involved 30 nucleotide sequences; positions containing gaps and missing data were eliminated to yield 112 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.[20]

2.5. Antigenic site modelling

Representative dimeric structural models for GI.2 and GII.4 capsid proteins were derived using an existing crystal structure of the Norwalk virus capsid protein as a structural template[21] and a full-length capsid sequence from the outbreak isolates, GI.2_Camp2_467, as well as a full-length GII.4 reference sequence, Hu/GII.4/New Orleans1805/2009/USA (gi:576106129), as target sequences.

Homology modelling was performed using a custom automodeller script based on the MODELLER engine with five rudimentary models generated and loop refinement performed on each model.[22] The best model for each target sequence was selected on the basis of DOPE quality scores.[23] Resulting models were structurally aligned in YASARA Structure using the MUSTANG algorithm, and structural and antigenic site comparison was performed.[24] Residue numbering of the GI.2 isolate was changed to GII.4 numbering in order to perform an accurate comparison of antigenic sites.

Informed consent was not required as this was an operational outbreak investigation, in line with institutional policy governing medical research. The use of the data and publication was approved by the Singapore Armed Forces Joint Medical Committee for Research.

3. Results

3.1. Camps and soldiers involved

Camp 1 was a camp with approximately 5000 soldiers spread across 21 groups, and camp 2 was a separate camp with about 700 soldiers spread across four groups. There were 150 to 250 soldiers in each group. A total of 720 soldiers from camp

1 were affected with AGE (15.0%) and 55 from camp 2 (8.3%) during the outbreak period.

In both camps, there were communal facilities shared by soldiers within a group (e.g., accommodation rooms and toilets), some by groups in close proximity (e.g., common training facilities) and some by all soldiers in the same camp (e.g., swimming pool). No direct interactions between soldiers in camps 1 and 2 could be identified prior to or during the AGE outbreak.

3.2. Community outbreaks

Over the weekend spanning September 14–15, two AGE outbreaks at restaurants in the local community involving 69 people were confirmed by the Ministry of Health, Singapore. During that time, about 3000 soldiers from 13 groups (including the index 'group A') in camp 1 and all soldiers from camp 2 were on home leave, although no direct link between the soldiers and the affected restaurants could be established.

3.3. Camp 1 outbreak

The outbreak epidemiological charts for camps 1 and 2 are shown in Figure 1. A chart depicting interactions of camp 1 soldiers affected during the first three waves is shown in Figure 2.

3.3.1. Wave 1

The first wave of AGE cases in camp 1 began on September 19, with 25 people from group A reporting sick at the medical clinic with AGE symptoms. At that time, group B, which usually had few interactions with group A, was in the same clinic for routine vaccinations.

3.3.2. Wave 2

Over the weekend of September 21–22, 13 groups returned home (including group A) and three groups were training out of camp. Of the five groups remaining in camp (1200 soldiers), groups C, D, and E had multiple interactions with group B at the common training ground, swimming pools, and dining areas (Figure 2). Fifteen soldiers from group D began reporting sick with AGE on September 22, followed by 96 soldiers across groups B, C, D, and E on September 24. Soldiers in group F, having had few interactions throughout, remained unaffected. The epidemiology department was notified on September 24, and control measures were promptly implemented.

3.3.3. Wave 3

The next wave of AGE cases began on September 26, involving 122 affected soldiers from six groups. The spread was likely through common living, training, and dining areas (Figure 2). To break chains of transmission in the closed setting, all soldiers returned home over the weekend of September 28–29, with health education reminders.

3.3.4. Plateau

On their return, an increased incidence of AGE cases was seen scattered throughout most groups over the next 5 days (September 30 to October 4), averaging 40 cases per day. Instead of the large clusters seen during the first three waves, cases were evenly distributed. Although routine cleaning principles including the use of chlorine bleach at 2500 ppm (within the US Centers for Disease Control and Prevention (CDC) recommended range) had been performed, a decision was made to strengthen the disinfection process, including increasing the concentration of chlorine bleach used to 5000 ppm (upper bound of the recommended range) for common touch points potentially soiled with bodily fluids. This occurred alongside a stepping up of public education on the touch points that could transmit disease.

3.3.5. Resolution

The number of soldiers reporting sick began to fall sharply from October 9, with an average of 10 cases over the next 3 days. By the following week, AGE cases in camp 1 had returned to baseline. No further waves were observed.

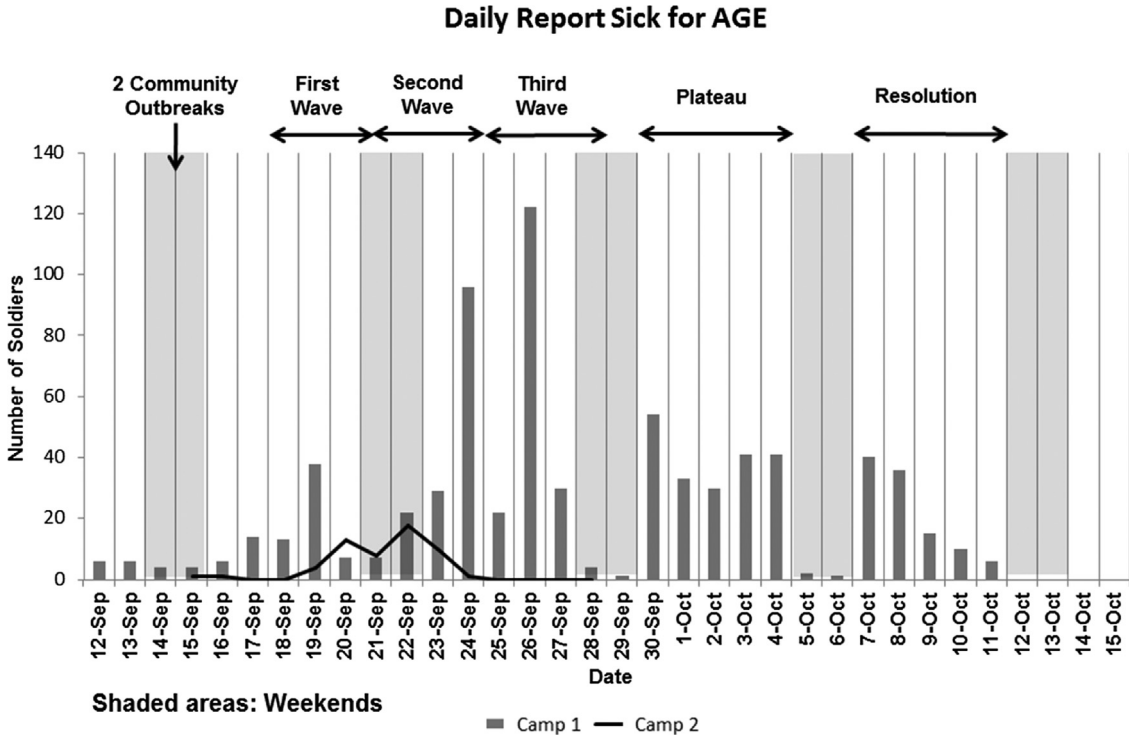


Figure 1. Epidemiological curve for camps 1 and 2 (by date of reporting sick).

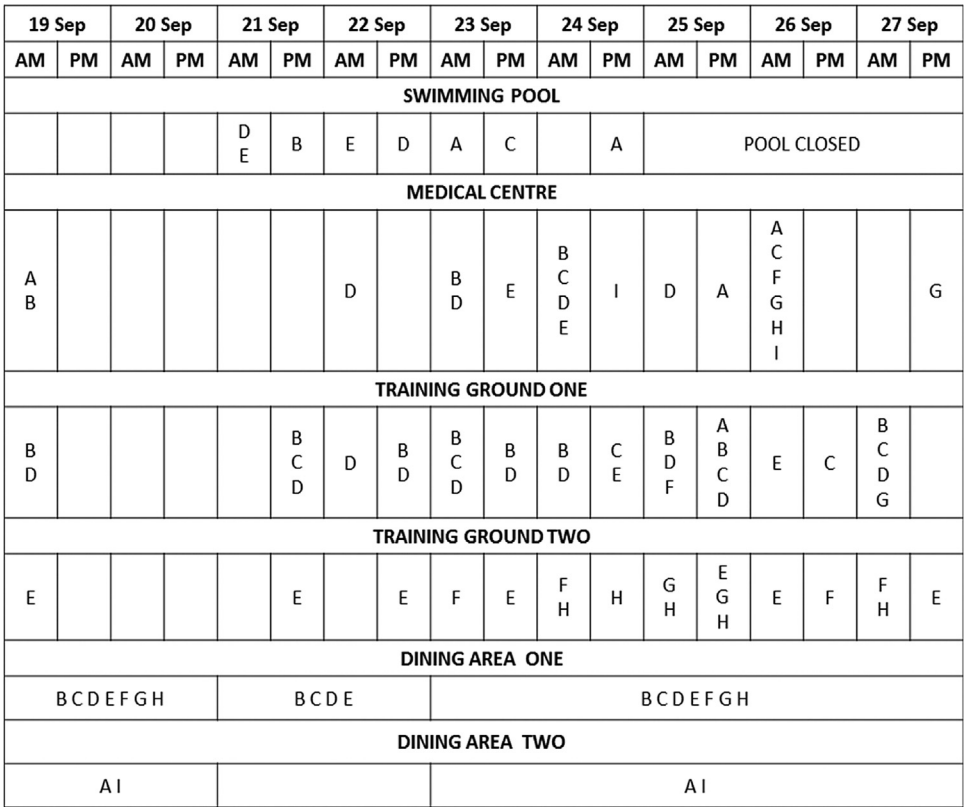


Figure 2. Activity chart for affected groups in waves 1 to 3 in camp 1. The various affected groups are labelled A to I. Over the weekend of September 21–22, groups A, F, G, H, and I were not in camp. The swimming pool and medical centre were common areas shared by all groups, whereas the training grounds and dining areas were only shared by a subset.

3.4. Camp 2 outbreak

The outbreak at camp 2 was smaller and resolved within 6 days.

3.4.1. Waves 1 and 2

The first wave in camp 2 began at the same time as that in camp 1, involving 17 soldiers reporting sick with AGE symptoms from September 19 to 20. This was followed by a second wave involving 28 soldiers on September 22 and 23. Likewise, once the epidemiology department was notified, outbreak control measures were instituted.

3.4.2. Resolution

Only sporadic AGE cases were seen thereafter. The last case was seen on September 24 and no further waves were observed.

3.5. Symptoms of affected soldiers

A symptom survey was conducted among 49 affected soldiers from camp 1 and 48 soldiers from camp 2; results are shown in Table 1. The predominant symptoms varied between camps, with more reports of diarrhoea in camp 2 (97.7% vs. 67.3%) and vomiting in camp 1 (75.5% vs. 50.0%).

Table 1
Distribution of symptoms in camps 1 and 2 from symptom surveys

Symptom	Camp 1 (%) (n = 49)	Camp 2 (%) (n = 48)
Vomiting	75.5	50.0
Nausea	71.4	70.8
Diarrhoea	67.3	97.7
Abdominal pain	57.1	85.4
Fever	22.4	33.3

3.6. Tests and other investigations

Food hall inspections, vocational fitness assessment of food handlers, testing of piped water, and food surveys all returned negative. Forty-eight hours of routinely stored food samples from camp 1 were sent for pathogen testing, although norovirus was not tested. One processed rice product returned positive for *Bacillus cereus*, but few soldiers had consumed the item, thus it was not likely related to the outbreak. Food samples from camp 2 had been inadvertently discarded before testing.

3.7. Stool sample testing

A total of 198 stool samples from cases in camp 1 and seven from camp 2 were tested, with 64 (32.3%) and two (28.6%), respectively, returning positive for norovirus GI. Of the remaining 139 samples from both camps, four returned positive for norovirus GII and the remaining 135 samples (65.2%) were undetermined. Full results are shown in Table 2. Stool samples of food handlers from both camps were also tested, returning undetermined. A decrease in positivity of samples to norovirus GI with outbreak resolution was also noted in camp 1. During the second wave, 16 of the 19 stool samples (84.2%) returned positive. This fell to 33.7% and 22.0% during the plateau and resolution phases, respectively. From October 10, there were no further positives.

3.8. Genomic analysis of norovirus GI stool samples

3.8.1. Phylogenetic analysis of norovirus sequences

Representative sequences (98% identity) from military and community (September 14–15) outbreaks and reference sets were aligned (**Supplementary Material**, Figure S1), and the relationship between these sequences represented as a phylogenetic tree

Table 2
Summary of stool testing results from affected cases

Pathogen	Camp 1	Camp 2
Norovirus I	64 (32.3%)	2 (28.6%)
Clostridium perfringens	8 (4.0%)	1 (14.3%)
Escherichia coli	8 (4.0%)	0
Norovirus II	4 (2.0%)	0
Campylobacter jejuni	1[a] (0.5%)	0
Bacillus cereus	1 (0.5%)	0
Shigella sp	1 (0.5%)	0
Salmonella sp	0	0
Listeria monocytogenes	0	0
Vibrio sp[b]	0	0
No pathogen detected	112 (56.6%)	4 (57.1%)
Total	198	7

[a] There was one case of two pathogens detected (Campylobacter and norovirus II).
[b] Vibrio species tested were Vibrio parahaemolyticus, Vibrio vulnificus, and Vibrio cholerae.

(Figure 3). The tree showed that the community and outbreak isolates were essentially identical to each other, as well as closely related to the closest representative BLAST hits from Asia and some from outside Asia on the basis of the sequenced regions; representative traditional GII.4 clinical isolates (blue) were phylogenetically distant.

No other GI genotypes aside from GI.2 were identified in the analysis. GII samples were detected sporadically along the course of the outbreak. As this was unlikely the cause of the outbreak, these were not sent for further genotype analysis.

3.8.2. Structural analysis of norovirus capsid antigenic regions

Given that the traditional outbreak strain in Singapore has been of the GII.4 genotype,[16] it is possible that antigenic differences compared to the GI.2 outbreak isolates in this study might have contributed to the observed pathogenesis in infected soldiers. Previous studies have examined the antigenicity of the norovirus capsid protein and identified specific regions of antigenicity. Structural alignment of the capsid models shows differences between the surface structures of the two models (Figure 4a and b), particularly in the loop regions where these antigenic regions have been shown to exist.[16] Examination of all known antigenic sites (Figure 4c) shows amino acid differences at multiple sites including regions A and C that lie prominently exposed on top of the molecule. This difference between the molecular surfaces at antigenic sites to previous outbreak strains (GII.4) suggests differences in antibody recognition, which may allow for unhindered proliferation of the GI.2 virus until immunity to the new strains builds up.

4. Discussion

Similar to other incidents in closed settings, the norovirus outbreak in the Singapore military camps spread quickly and widely due to communal living and training environments – a

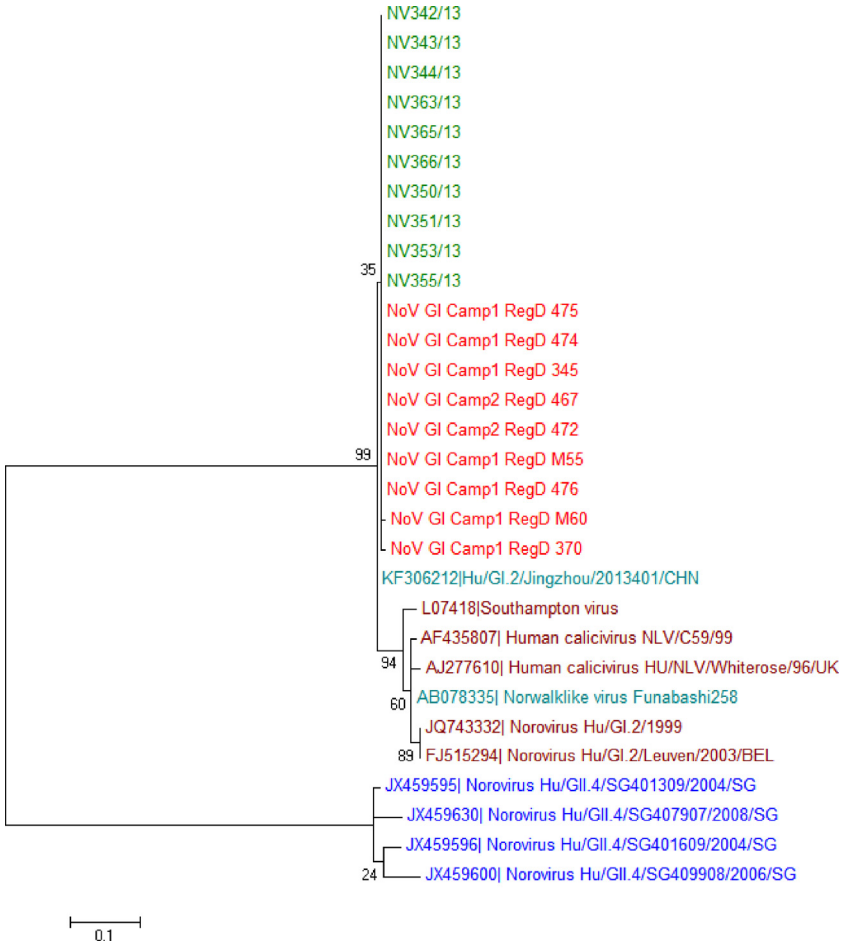
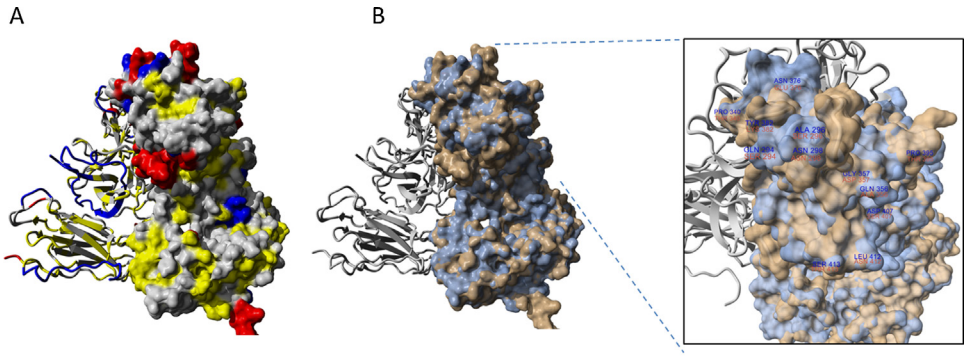


Figure 3. Phylogenetic relationship between norovirus isolates. The isolates from the outbreaks (red) were compared to community isolates from the period preceding the outbreaks (green), closest BLAST hits from Asia (teal), closest BLAST hits from outside Asia (maroon), and representative GII norovirus (blue). Phylogenetic relationships were inferred using the maximum likelihood method in MEGA6. One thousand bootstrap replicates were performed and the tree with the highest log likelihood is shown.



C

	A			C	E			A	C	B	D		E		
	294	296	298	340	356	357	372	376	382	393	395	407	412	413
GII.4 New Orleans 2009	S	R	N	T	A	D	D	E	K	S	T	S	N	T
GI.2 Camp2_467 2013	A	R	T	P	Q	G	D	N	Y	S	P	D	L	S

Figure 4. Comparison of antigenic sites between GII.4 and GI.2 representative capsid models. (a) Best-scoring models representative of the GI.2 capsid (blue) and the GII.4 capsid (red) were generated using the MODELLER program and overlaid in YASARA to highlight structural and sequence identity (yellow) and structural identity but different amino acids (grey), as well as strong structural differences shown in the original surface colours of the models. (b) Antigenic site comparison between GI.2 and GII.4 capsid proteins. The overlap between the molecular surfaces of GI.2 (blue) and GII.4 (orange) suggests differences in antigenic sites where putative antibodies might bind. A summary of the residue differences at all known antigenic sites between the two capsid representations is shown in (c). Residue clusters forming epitope regions are denoted above the table, from A to E.

persistent problem for the military worldwide.[8,9,25–28] The attack rates of 15.0% and 8.3% in camps 1 and 2, respectively, are comparable to those of previously reported norovirus AGE outbreaks in military settings, which have ranged from 10.4% to 12.4%.[7,28] These attack rates are lower than those reported from outbreaks in other closed settings, where the incidence has ranged from 20% to 53%,[29] especially in hospitals and cruise ships.

Population-level control measures at camp 1 were implemented after 136 people across five groups had been affected (wave 2), during which soldiers affected in the subsequent wave were likely in the incubation period. The effects of mitigating actions were therefore only effective after the subsequent wave had abated. This highlights the need for improved AGE surveillance systems to detect early signs of impending or evolving outbreaks. Systems in use globally include internet-based passive reporting systems,[30] and syndromic surveillance from telephone triage and pharmacy data.[31] For example, CaliciNet, a national norovirus outbreak surveillance network that uses electronically submitted laboratory data, detected a new GII.4 variant in 2009, which subsequently caused 60% of AGE outbreaks that winter.[32] Such systems may have merits in closed settings, since they allow for mitigation of local outbreaks through early action. The Singapore military has since implemented sentinel surveillance and laboratory testing of a proportion of daily AGE cases and real-time recording of all AGE cases to identify outbreak prodromes. Additional outbreak thresholds for camps are also being modelled from historic baseline AGE rates, while automated surveillance via electronic medical record systems is being developed. Such measures will help to identify future outbreaks earlier.

The differences between the two AGE outbreaks highlight difficulties in controlling person-to-person outbreaks among large groups in close proximity (e.g., military trainees) once substantial spread has taken place. Although both camps informed the epidemiology department at the same time, camp 1 was a camp with a larger base population and multiple shared facilities serving as points of contact for the rapid transmission of disease. Soldiers in camp 1 also presented with more vomiting, which may have led to more efficient propagation.[33]

Although the transmission of norovirus between fingers and surfaces,[34,35] and the efficacy of chloride-based solutions in its inactivation have been described previously, a strong evidence base for most outbreak control recommendations remains poor,[36,37] with the exception of proper hand hygiene.[38] Although increased viral loads on high contact objects have been described, there is no good evidence regarding the efficacy of commonly used disinfection methods on such surfaces. There is thus a need for further studies on fomite transmission models and disinfection optimization.

The genetic similarity between noroviruses from both community and military outbreaks is reflective of the close interactions between the local populace and the military in Singapore, especially when significant proportions of soldiers return home regularly. Previous reports of similar spread involving other diseases have been published, including a hepatitis A outbreak in the US military linked to a childcare facility and an outbreak of mumps among military personnel in Luxembourg.[39,40] This also emphasizes the value of close collaboration between the military and community epidemiological elements.

There were concerns as to whether soldiers returning home would become index cases for further cases of norovirus gastroenteritis in the general community. To prevent this, soldiers were reminded of the need to maintain personal hygiene. Rates of diarrhoeal illness at government clinics in Singapore were monitored and there were no significant increases in incidence during the immediate preceding or subsequent time periods.[41] This further suggests that any secondary spread from the military to the community was limited.

Finally, the two outbreaks described represent the first documented GI.2 outbreaks in Singapore. A study by Lim et al. showed that all of the 37 documented national outbreaks from 2004 to 2011 were due to norovirus GII.[16] This deviation from the traditional outbreak-associated GII.4 strain may have resulted in a greater proportion of soldiers being immunologically susceptible to falling ill with AGE and contributing to the initial exponential propagation. This is supported by the homology modelling, which showed distinct differences in antigenic sites between GII.4 and GI.2. Although much of the norovirus vaccine development has

been centred on the prevention of GII.4, such outbreaks are reminders of the outbreak potential of other emerging genotypes as well.

There are limitations to this study. The number of soldiers affected included baseline AGE disease cases that may not have been directly related to the ongoing norovirus outbreak. A portion of these may have been captured under the 11.5% of stool samples positive for other pathogens. The contrary is also true – soldiers who had sought medical consultations outside of the military medical system during their off days were not captured, and milder cases that did not fulfil the AGE criteria were not counted as part of the outbreak. Furthermore, as we did not incorporate internal amplification controls in the PCR reactions, samples that did not return positive could not be determined conclusively as 'true' negatives, regardless of the pathogen.

As a direct link between preceding community outbreaks and the subsequent military outbreaks could not be identified, the association between the two remains circumstantial and based on the temporal chain of events. While the phylogenetic evidence suggests that the community and subsequent military outbreaks were linked in terms of a common novel circulating norovirus lineage, the direction of transmission of the outbreak isolate could not be ascertained. Furthermore, the isolate Hu/GI.2/Jingzhou/2013401/CHN collected in April 2013 (Figure 3) is evidence that this lineage had been circulating regionally, adding further uncertainty to the actual source of transmission.

In conclusion, we have described the epidemiological and phylogenetic characteristics of two concurrent norovirus GI.2 outbreaks in the Singapore military likely linked to preceding local community outbreaks. Although both are semi-closed, communal settings, differences in attack rates between the two indicate a greater propensity for transmission within large camps with a high population density and contained spaces. However, these settings also provide opportunities to study transmission patterns, as well as to develop improved surveillance systems and control measures.

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Ethical approval: This study was approved by the Singapore Armed Forces Joint Medical Committee for Research.

Conflict of interest: The authors have no conflicts of interest to declare and there were no external sources of funding or sponsorship for this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2014.12.023.

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