Inactivation of murine norovirus on a range of copper alloy surfaces is accompanied by loss of capsid integrity

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Running head: Norovirus inactivation on copper

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Norovirus is one of the most common causes of acute viral gastroenteritis. The virus is spread via faecal oral route, most commonly from infected food and water, but several outbreaks have originated from contamination of surfaces with infectious virus. In this study a close surrogate of human norovirus causing gastrointestinal disease in mice, murine norovirus type 1 (MNV-1), retained infectivity for more than 2 weeks following contact with a range of surface materials including Teflon (polytetrafluoroethylene, PTFE), polyvinyl chloride (PVC), ceramic tiles, glass, silicone rubber and stainless steel. Persistence was, slightly prolonged on ceramic surfaces. A previous study in our laboratory observed that copper and copper alloy dry surfaces rapidly inactivated MNV-1 and destroyed the viral genome. In this new study we have observed that a relatively small change in percentage copper, between 70-80% in copper nickels and 60-70% in brasses, had significant influence on the ability of the alloy to inactivate norovirus. Nickel alone did not affect virus but zinc did have some antiviral effect which had a synergistic effect with copper and resulted in increased efficacy of brasses at lower percentage copper. Electron microscopy of purified MNV-1 that had been exposed to copper and stainless steel surfaces suggested a massive breakdown of the viral capsid had occurred on copper. In addition, MNV-1 that had been exposed to copper and treated with RNase demonstrated a reduction in viral gene copy number. This suggests capsid integrity is compromised on contact with copper, allowing copper ion access to the viral genome.
Noroviruses are responsible for approximately half of all cases of gastroenteritis worldwide. The low infectious dose, ability to persist in an infectious state in the environment and resistance to many commonly used cleaning agents has led to many disease outbreaks that have proved very difficult to contain [1][2]. The virus is spread directly via the faecal-oral route but also from touching contaminated surfaces which has recently been found to be more significant than originally thought in the spread of many diseases [3]. Ineffective cleaning agents may leave residual virus particles on surfaces which can initiate an infection [4]. Norovirus disease is usually self-limiting with symptoms lasting a few days but can be more serious in severely ill or immunocompromised individuals especially if the causative agent is one of the emerging recombinant strains, including GII.g/GII.12, which appeared in 2008 and has enhanced virulence and severity of clinical symptoms. Asymptomatic carriage and extended virus shedding also increase the risk of transmission [5] [6]. A recent study of a large waterborne outbreak in Nokia, Finland, also observed that norovirus exposure may result in more long term health effects which persisted 15 months after the initial infection [7]. This may mean that the considerable public health costs incurred in initial outbreaks, estimated in the US to be more than $2 billion per year, may just be the tip of the iceberg if the virus is responsible for long term pathologies.

The use of antimicrobial surfaces in high risk environments may help to prevent the spread of many infectious agents that are able to retain infectivity on surfaces. Copper and copper alloys have been shown to be effective at rapidly killing a range of bacterial, fungal and viral pathogens in laboratory studies at a range of temperatures and humidity conditions [8], [9], [10], [11], [12], [13]. This has led to clinical trials incorporating copper surfaces in busy wards where reduction in bioburden and infection rate have now been observed in rooms.
equipped with just a few copper surfaces. [14], [15], [16], [17]. The mechanism of action has also been shown to be complex and variable, involving release of copper ions from the surfaces which have a direct action and/or lead to generation of secondary agents of toxicity such as reactive oxygen species which affect a variety of targets [18,19]. We have shown previously the rapid destruction of murine norovirus, a close surrogate for the human norovirus causing gastrointestinal disease which is necessary because of the lack of suitable infectivity assay for the human norovirus, on a small range of copper alloys [20]. This study observed that the rate of inactivation was affected by temperature and aqueous content, and did not involve generation of reactive oxygen species. The current study has continued this work investigating a larger range of copper nickel and brass alloys, including the effect of surface texture and nickel and zinc controls. The persistence of a range of commonly used non-metal surfaces was also investigated. The previous study observed that exposure to copper resulted in destruction of the viral positive strand RNA genome. In this study the effect on the viral capsid was investigated.

Materials and methods

Murine norovirus (MNV-1) and cell lines:

Acquisition, preparation and maintenance of virus stocks and cell lines have been described previously [20]. MNV-1 CW1, and the mouse monocyte macrophage line, RAW 264.7, were kindly supplied by Professor Herbert Virgin IV, Washington University, US.

Preparation of purified MNV-1

A preparation of purified MNV was prepared by adapting the method of Wobus et al [21]. Briefly, RAW 264.7 cells were infected at multiplicity of infection (MOI) of 2 and incubated
at 37°C 5% CO₂ for 48 hours when significant cytopathic effect (CPE) was visible. Following 3
freeze/thaw cycles the virus was precipitated out of the cell lysate with polyethylene glycol
(BioVision PEG Virus Precipitation Kit) and further purified by caesium chloride isopycnic
 centrifugation. The virus band was aspirated and dialysed overnight against PBS. The virus
was then concentrated by pelleting through a sucrose cushion (30% (w/v) sucrose) at 90,000
x g for 2.5 hours at 4°C in Beckman Coulter L7 65 ultracentrifuge; 100 μL ice cold PBS was
added to the virus pellet and incubated on ice for 30 minutes. The virus was re-suspended
by gentle pipetting and stored at -80°C until required.

Preparation of sample surfaces:

Metal coupons with a surface area of 1cm² and thickness 0.5mm were degreased in acetone
(to remove any lipid film which may delay the antimicrobial effect of copper([22]), stored in
absolute ethanol and flamed before use as described previously (20). The constituents of
each metal tested are detailed in Table 1 were supplied by the Copper Development
Association.

Non-metal surfaces (Table 2, Teflon (polyfluorotetraethylene, PTFE), polyvinyl chloride (PVC),
ceramic tiles, glass, silicone rubber) were also cut into 1cm² coupons and were sterilised by
autoclaving. Metal controls for comparison were also autoclaved for method consistency for
these experiments.

Investigations increasing surface roughness of metal coupons were performed on some
alloys. Coupons were abraded by rubbing with coarse sandpaper for 5 minutes, rinsed well
in DDW and checked with EDIC microscopy that no grains remained on the coupons. The
coupons were then prepared for surface testing as described.
Plaque assay for infectious virus recovered from test surfaces:

Stock cell lysate preparations of MNV-1 were spread over the surface of the coupons for at room temperature (approximately $5 \times 10^5$ plaque forming units (pfu) in 20 μL per coupon). This is 10-50 x more virus than was used in the previous study where the virus was diluted in cell growth medium for comparison between dry touch and wet fomite simulated contamination (20). This new study has investigated simulated wet fomite surface contamination only. The virus was removed from the coupons at required times and assayed for infectivity in RAW 264.7 cells as described previously (20). The method resulted in a recovery rate of > 95% inoculum.

Morphology of purified MNV-1 recovered from metal surfaces

The purified virus was inoculated onto copper and stainless steel coupons (1cm²) and incubated for 2 hours at room temperature. The virus was removed from the coupons by gentle pipetting in sterile, nuclease-free distilled deionised water. 5 μL preparations of virus exposed to metal surfaces or untreated virus were dried on to copper grids and stained with the negative stain phosphotungstic acid (PTA) or ammonium molybdate for 5s and observed using a transmission electron microscope.

Determination of capsid integrity by pre-treatment with RNase

MNV was inoculated onto 10 replicate coupons for copper or stainless steel as described for surface testing. Virus was removed immediately or after 2 hours contact by pipetting in as small a volume of PBS as possible (200 μL). Half of the sample was treated with 1 μL RNase plus 11 μL RNase buffer in Promega RNase ONE Ribonuclease kit for 15 minutes at 37°C. Viral RNA preparations and cDNA were prepared and real time PCR amplification of viral
gene important in early stages of infection (VPg) was performed on untreated and RNase treated samples as described previously (20). Exposure to copper surfaces has been shown to result in massive degradation of the genome suggesting the results could apply with any viral gene (20).

**Statistical analysis**

Data are expressed as mean ± standard errors of the mean (SEM) and are from multiple independent experiments. Differences between duplicate samples were assessed using the Mann-Whitney rank t-test. Group comparisons were analysed using the Mann-Whitney U test where statistical significance was expressed as p < 0.05. Statistical analyses and graphical representations were performed using Sigma Plot version 12.5 and GraphPad Prism 6.

**Results and discussion**

Infectious MNV-1 retains infectivity on non-metallic surfaces for several weeks at room temperature.

A 1-log reduction in infectivity was observed over the first 5 days of exposure to the non-metallic surfaces: Teflon (polyfluorotetraethylene, PTFE), polyvinyl chloride (PVC), ceramic tiles, glass, and silicone rubber (Figure 1). Between 5-14 days there was a steady reduction in infectivity but given that the infectious dose is very low, approximately 10 virions, this means there is a considerable risk of infection transmission from all surfaces tested at 2 weeks. In this test the inoculating concentration was approximately 5 x 10^5 pfu per cm² test surface. This concentration is similar to that expected from surface contamination by vomitus which can contain up to 10^7 virus per 30 mL. However, the faeces of an infected individual may contain up to 10^{11} virions per g, and survival of infectivity on faecal
contaminated surfaces could be considerably longer than reported here [23]. It is interesting to observe that the highest levels of infectivity were retained on ceramic tiles which could be significant as this material is often employed in bathrooms and kitchens where majority of norovirus contamination will occur.

Inactivation of MNV-1 on copper nickels

Our previous study suggested copper nickel, C70600, was very efficacious at inactivating MNV-1, even when compared to phosphor bronze - an alloy which contains 6% more copper. In this current study a further 3 alloys were tested (Figure 2). MNV-1 was rapidly inactivated on alloys containing 79 to 89% copper but loss of efficacy at 70% suggesting a small difference in copper content, 70-79%, can have a large effect on antiviral efficacy. The greatest difference in inactivation rate occurred over the first 30 minutes contact (Figure 2B) where C71000 had a very similar inactivation rate to pure copper and C70600. The exception to this was alloy C72500 that had reduced efficacy compared to C70600 which had the same copper content. The appearance of these alloys is very different: C72500 is very shiny and polished compared to the dull C70600. This may be due to a passivating oxide layer which may contribute to efficacy (this alloy is employed in marine engineering because of excellent antifouling properties). Hans et al(2014) [24] reported a layer of cuprous oxide (Cu$_2$O) was as effective at killing Enterococcus hirae as pure copper but found copper II oxide, CuO, to be inhibitory by forming a protective layer reducing the rate of corrosion (passivation layer). We have observed that if the surface of alloys C70600 and C72500 were abraded by rubbing with coarse sandpaper (Figure 3A) the efficacy of the latter to inactivate MNV-1 was reduced following 30 minutes contact at room temperature (Figure 3B). This suggests the presence of an oxide layer on this alloy contributes to the...
antiviral efficacy. By contrast, abrading the surface of copper, stainless steel and alloy C72500 did not have any effect on their untreated antiviral efficacy. In the previous study (20) phosphor bronze was not as effective as alloys with lower % copper and this alloy contains 5% tin. Alloy C72500 contains 2% tin and it is unclear if the presence of tin has an inhibitory effect on copper ion release and therefore reduces the ability of copper to inactivate norovirus. The shiny polished surface of our samples also exhibited increased hydrophobicity when virus was inoculated onto the metal surface. Although the inoculating virus was 10-50 times higher than in previous study, MNV-1 was inactivated in a shorter time on C70600. Perhaps the increased concentration of cellular debris, as well as virus, has affected results. Stainless steel and nickel did not have any antiviral activity suggesting the inactivation of MNV-1 was due primarily to copper.

Inactivation on brasses and influence of zinc

Our previous study suggested that infectivity of MNV-1 was also reduced on cartridge brass, C26000. In this new study, using a viral inoculum containing a higher concentration of virus and RAW264.7 cell debris, MNV inactivation was a little faster and totally inactivated at 2 hours. The other alloys demonstrated MNV inactivation directly proportional to percentage copper (Figure 4). However, over a 1-log_{10} reduction in MNV infectivity was observed on pure zinc. Comparison between copper-nickel and brass which both contain 70% copper demonstrate the increased efficacy of brass (Figure 5) which may be due to the synergistic action of copper and zinc to inactivate MNV. Zinc was first reported to inhibit replication of rhinoviruses in 1974 [25] and subsequently zinc salts and lozenges have been used as therapies for the common cold and influenza. More recently the antiviral effects of zinc ionophores, such as pyrithione, have been investigated. Increased uptake of Zn^{2+} through...
the ionophore results in inhibition of herpes simplex virus (HSV) (a large enveloped double-stranded DNA virus) replication by deregulating the ubiquitin-proteasome system (UPS) and activating the NF-κB (nuclear factor kappa-light chain enhancer activated B cells)[26]. Here a small change in % copper in the alloys tested (70-80%) had a large effect on efficacy to inactivate MNV. C28000 had the lowest copper content (60%) and a similar efficacy to pure nickel, despite the higher zinc content of 40%. All other copper alloys tested were very effective, totally inactivating $5 \times 10^5$ pfu per cm$^2$ within 2 hours at room temperature.

A recent study has found that a zinc oxide passivation layer developed on brass surfaces in eccrine fingerprint sweat within an hour [27]. It is yet to be determined if this could affect the antiviral efficacy of brass.

**Exposure to copper and copper alloy surfaces damages the norovirus capsid.**

Observation by TEM of purified MNV-1 suggested that some damage to the capsid had occurred during the purification procedure (Figure 6). The outer surface of the capsid was uneven and irregular (Figure 6A). However, the purified fraction was found to be still infectious in the plaque assay demonstrating the resilient nature of the capsid to protect the infectious viral RNA. Virus exposed to stainless steel was visible as individual particles (Figure 6B) or had aggregated into large clumps of approximately 100 nm diameter (Figure 6C). This may offer some protection to virus particles within the aggregates and explain persistence in an infectious state for weeks. The aggregation may be affected by the absence of cell debris and PBS diluent.

Purified MNV exposed to copper surfaces appeared to have disintegrated into fractions beyond the resolution of the TEM (Figure 6D). Our previous study suggested release of
Cuprous and cupric ions were essential for antiviral activity, including destruction of the viral genome. The TEM results suggest extensive damage to the viral capsid must have occurred presumably as a result of direct or indirect action of copper ions. It is unclear if capsid damage is a result of multiple, non-specific attacks to the capsid surface or a dissociation of the capsomeres to expose the viral RNA.

Comparisons between MNV removed from copper and stainless steel surfaces that have been treated with RNase support this premise (Figure 7). Virus removed from stainless steel surfaces immediately after inoculation did not demonstrate any difference in copy number of viral gene, Vpg, regardless of pre-treatment with RNase. This suggests the capsid is intact, with no exposed viral genome for RNase degradation. However, if virus is removed immediately from copper surfaces the copy number is reduced in the RNase treated sample. This suggests that damage to the capsid has already occurred in the time it takes to process the sample (although infectivity is not reduced significantly at Time 0). After 2 hours contact overall copy number is reduced on copper but the reduced amplification of VPg on both surfaces suggests capsid damage had occurred on copper and stainless steel but not so extensively on the latter. In our original work we observed that if the virus is allowed to dry rapidly infectivity was reduced much faster because the rapidly drying process affected virus infectivity.

Potential application

The highly infectious nature of norovirus makes it very difficult to stop the spread of infection from an outbreak especially as no vaccine or treatment is available and the infection is often passed onto the carers of the infected individuals. Wheeler et al estimated that for every single case reported in the UK this leads to 136 in the community. Morter et
al., 2011, detected norovirus using real time RT-PCR in 31% of more than 30 surfaces tested in a hospital environment over a 4 month period with 100% contamination on soap and alcohol dispensers, chairs, carpets and Zimmer frames [28]. Our results suggest that incorporation of copper alloy surfaces may help to prevent infection spread from contaminated surfaces. In many norovirus outbreaks reported, contaminated surfaces are responsible for a secondary outbreak that occurs primarily because of inadequate disinfection of vomitus and faecal norovirus contamination. For example, following an initial outbreak in a hospital ward 2 carpet fitters contracted norovirus 12 days later when replacing the flooring in the outbreak area [29]. It is very difficult to reduce spread of norovirus infection from person-to-person in an initial outbreak where segregation and quarantine of infected persons may be the only way to halt the spread of infection, although this is not always practical. There is a need for more effective and safe disinfectants for decontaminating large areas of norovirus contamination. Incorporation of copper alloys could be very useful in preventing secondary transfer from ineffectively cleaned and highly contaminated surfaces in clinical facilities, closed environments such as cruise ships, care homes, kitchens, bathrooms, surfaces.

There is now a large body of evidence describing the importance of copper alloy touch surfaces as antibacterial surfaces, which have been supported by hospital studies worldwide showing reduced bioburden on touch surfaces and decreased rate of infection in ICUs. Latterly we have demonstrated copper alloy antiviral activity against the enveloped and non-enveloped RNA viruses, influenza and norovirus, respectively. We now report that the mechanism of copper inactivation for norovirus involves not only degradation of the RNA but also destruction of the capsid which allows copper ions to enter the virus. These studies
with RNA viruses suggest that copper alloy surfaces might exhibit antiviral activity against other important RNA viruses where transmission via touch surfaces is important, including coronavirus (unpublished data) and Ebola virus.

Acknowledgements

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Figure legends

Figure 1 Persistence of infectious murine norovirus on common surface materials
Approximately 5 x10^5 pfu infectious virus was applied to 1cm^2 samples of test surfaces and incubated at room temperature. At required time points virus was removed from the surfaces and assessed for infectious virus by plaque assay as described in the text. No significant reduction in infectivity of norovirus occurs on any surface over 2 hours at room temperature. This was followed by a steady decline in infectivity of norovirus: Infectious virus was present on all surfaces except stainless steel and silicon rubber at 20 days. However, because the infectious dose is very low, only 10 virus particles, this represents a considerable risk of infection spread. Slightly higher levels of infectious virus were recovered from ceramic surfaces commonly used in bathroom and kitchen tiles. (PVC blue ( ), Teflon red (■), ceramic green (▲), glass purple (▽), Silicon rubber orange ( ), stainless steel S30400 black ( )) . Error bars represent ± SD and data are from multiple experiments.

Figure 2 Inactivation of MNV on copper nickels
A: Survival of MNV infectivity on copper nickels

Approximately $5 \times 10^5$ pfu infectious virus was applied to 1 cm$^2$ samples of test surfaces and incubated at room temperature. At required time points virus was removed from the surfaces and assessed for infectious virus by plaque assay as described in the text. MNV was rapidly inactivated on copper and C70600. The extent of inactivation on copper alloys was proportional to % copper EXCEPT alloy C72500 which is not as effective as alloys with lower %. Stainless steel and nickel do not have any antiviral activity.

B: Rate of MNV inactivation on copper nickels over the first 30 minutes contact at room temperature

The inactivation rate, K, was calculated as described previously (20). C11000 (100% copper), C70600 (89% copper) and C71000 (79% copper) all displayed similar, fast inactivation of MNV for the first 30 minutes contact. Inactivation on C25000 was slower even though copper content is high (89%).

Figure 3 Comparison between two copper nickels, C72500 and C70600) containing 89% copper

A: appearance of copper alloys: abraded and un-abraded (copper and stainless steel controls)

B: Abrasion of oxide layer on C70600 slightly reduces antiviral efficacy

Approximately $5 \times 10^5$ pfu infectious virus was applied to 1 cm$^2$ samples of abraded and un-abraded metal coupons and incubated at room temperature. At required time points virus was removed from the surfaces and assessed for infectious virus particles by plaque assay as described in the text. Abrading the surface of alloy C72500 has not made any difference in efficacy to inactivate MNV. Is it unclear if the tin content of this alloy affects efficacy.
Abrading the surface of alloy C70600 reduces antiviral efficacy i.e. the alloy surface has an effect on MNV.

Figure 4 Persistence of MNV infectivity on brasses
Approximately $5 \times 10^5$ pfu infectious virus was applied to 1 cm$^2$ samples of test surfaces and incubated at room temperature. At required time points virus was removed from the surfaces and assessed for infectious virus by plaque assay as described in the text. The efficacy of these brasses to inactivate norovirus is directly proportional to the copper content; 100% Zn appeared to have some antiviral activity similar to Muntz metal (C28000). The possible antiviral effect of zinc is discussed in the text.

Figure 5 Comparison between ability of brass and copper nickel with the same % copper (70%) to inactivate MNV
Brass is more effective at inactivating norovirus (the effect is more evident after 30 minutes contact) presumably due to the synergistic action of copper and zinc.

Figure 6 Contact with copper surfaces affects virus morphology
A: untreated virus. The uneven surface suggests some damage has occurred to the outer capsid resulting in a diameter slightly larger than 40 nm reported for individual virions and disrupting the icosahedral symmetry. Plaque assay of this preparation suggests virus is still infectious so the genome is still able to replicate. This demonstrates the protective nature of the capsid and its role in MNV persistence.

B and C: MNV recovered from stainless steel was observed as scattered individual particles (B) or in large clumps (C) that had some damage to the outer capsid. Viral clumping may protect the inner virus particles from desiccation and explain persistence on this material.
D: In contrast exposure to copper resulted in viral fragments that were beyond resolution of the TEM Bar 200 nm

Figure 7 Pre-treatment of test samples with RNase to determine capsid integrity

If MNV is incomplete or the capsid is damaged the viral nucleic acid may be exposed and susceptible to RNase treatment. Approximately 5 x10^5 pfu infectious virus was applied to 1 cm^2 samples of test surfaces and incubated at room temperature. At required time points virus was removed from the surfaces. Viral RNA was purified from the samples, cDNA prepared and assessed for viral genome by RTqPCR as described in the text. Copy number was derived from standard curve of standard VPg supplied by PrimerDesign Ltd. Virus exposed to copper appears to have suffered damage to capsid immediately on contact because VPg copy number is reduced in RNase treated samples suggesting viral genome was exposed. This has not occurred on stainless steel at Time 0 suggesting that intact capsid is impervious to RNase. After 30 minutes contact capsid damage is seen for both surfaces which may be affected by drying process as well as the antiviral effects of copper.
Table 1 Constituents of metals used in the study

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Table 2 Range of commonly used surfaces to investigate the persistence of MNV-1 at room temperature
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| 2      | PVC (polyvinyl chloride) | Low cost (replace expensive metals), hard but can be made flexible with plasticizers | 3rd most used plastic, construction, (pipes, window) 2800-3000, flexible,
| 3      | Ceramic tiles    | Glazed Sr, Na, K, Ca oxides              | Tableware, bathroom, kitchen tiles |
| 4      | glass            | 75% silicon dioxide, 1 Na, Ca oxides    | Ubiquitous because of transparency and non-reactivity |
| 5      | Silicone rubber  | Low density, elastomer, heat resistant   | Seals, cooking utensils, baking sheets |
| 6      | Stainless steel 100.100 | Resistant to alkaline, apparently easy to clean | Stylages, stove, automotive |
Survival of MNV infectivity on common surface materials

- PVC
- teflon
- ceramic
- glass
- silicon rubber
- stainless steel metal

Log 10 pfu per coupon vs. Contact time (days)
Survival of MNV infectivity on 70% copper alloys

- C71500 (70% Cu) copper nick
- C26000 (70% Cu) brass