

**Virucidal activity of acids and disinfectants against
pathogenic viruses**

酸および消毒薬の病原性ウイルスに対する

不活化効果の検証

Joint Graduate School of Veterinary Medicine

Yamaguchi University

Hu Weiyin

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1. General introduction

Enveloped viruses

1.1. Transmissible gastroenteritis virus (TGEV)

History

Transmissible gastroenteritis virus (TGEV) was first identified in 1946 by Doyle and Hutchings in the United State [1] . TGEV has also been reported in European, Americas, and Asia, causing significant economic losses in the swine industry worldwide. According to Monika Olech et al [2], TGEV infection in swine are rare in Europe but more prevalent in the Americas and Asia . In Korea, Oh et al. [3] analyzed 1,295 serum from slaughtered pigs and 69 sera from wild boars collected between 2009 and 2010, finding that 4.9% of the samples tested positive for TGEV antibodies. In Japan, Miyazaki et al. [4] examined TGEV antibody prevalence across six regions, including Tohoku, Hokuriku, Kanto, Chubu, Chugoku, and Kyushu, and reported 12.5% positive sera for TGEV antibody using ELISA, and 14.4% by virus neutralization test .A review by Chen et al. [5] reported the overall TGEV prevalence in China at 10% , with a notably higher prevalence of 38% in the northeast region . In addition to the detection of TGEV, natural variants have also been isolated. In China, three virulent strains with strong pathogenicity have been reported, including strain JS2012 in 2020 [6], strain HQ2016 in 2016 [7] , SC2021 in 2021[8] . These mutant strains have been associated with high mortality rates and severe gastrointestinal signs. In Japan, strain TO163 isolated from Tohoku District in 1999 and has since served as a reference strain in multiple seroepidemiologic and molecular studies.

Virus properties

TGEV is an enveloped, single-stranded positive-sense RNA virus belonging to the genus *Alphacoronavirus* in the family *Coronaviridae* [9]. Its genome is approximately 28.5 kb in length and encodes both non-structural and structural proteins [10]. TGEV has four major structural protein: spike glycoprotein (S), envelope (E), membrane protein (M). S protein mediates viral attachment and entry into host cells. E protein involves in virus assembly, budding and pathogenesis. M protein

involves in virion morphogenesis and assembly. TGEV interact with aminopeptidase N (APN) as a functional receptor.

Disease and pathogenesis

TGEV infection exhibits significant age-dependent signs in swine with the severity of clinical signs and outcomes of infection varying significant across different age groups [11] . Piglets under two weeks of age often develop acute gastrointestinal disease, leading to severe watery diarrhea with 50–100% mortality [9] . TGEV specifically targets and destroys the mature enterocytes lining the villi of the small intestine, resulting in villous atrophy, malabsorption, and severe watery diarrhea [12] . In contrast, TGEV-infected adult pigs exhibit mild or subclinical infection. While they may show transient signs such as reduced appetite or mild diarrhea, these are generally self-limiting and do not result in significant mortality. Nevertheless, subclinically infected adults pig can serve as asymptomatic carriers, contributing to viral persistence and transmission within herds [13] .

Treatment and prevention

No specific antiviral treatment is available for TGEV. Management focused on treating clinical signs. According to the World Organisation for Animal Health (WOAH), vaccination is practiced in only a limited number of countries, such as United States of America [14]. Sows and gilts that are either infected or vaccinated against TGEV can develop specific protective epitopes, induce lactogenic immunity and offer protection to piglets [15]. Nevertheless, systematic monitoring and surveillance are essential for effective disease prevention and control. Public sanitation is critical in managing TGEV outbreaks. Conventional serological tests such as virus neutralization, indirect fluorescent antibodies, agar gel precipitation, radioimmunoprecipitation, indirect immunoperoxidase and enzyme-linked immunosorbent analysis (ELISA) tests are used for TGEV detection [2] .

1.2. Porcine epidemic diarrhoea virus (PEDV)

History

Porcine epidemic diarrhoea (PED) was first identified as a distinct enteric disease in pigs in the United Kingdom and Belgium in the early 1970s [16]. The causative agent, Porcine epidemic diarrhoea virus (PEDV), strain CV777, was isolated in Belgium in 1977 and classified as a new coronavirus, Porcine epidemic diarrhoea virus (PEDV) [17]. The virus spread across Europe during the 1970s and 1980s [18]. In Japan, PEDV was first detected in 1982–1983 [19,20], with large-scale outbreak in 1993–1996 [21,22] causing high piglet mortality. A live vaccine approved in 1996 [21] led to sporadic cases until a major resurgence in 2013 despite ongoing control measures [23]. In China, PEDV was detected in 1980s and remained a mainly regional problem while CV777-based bivalent vaccines were widely used. However, the emergence of highly virulent variant strains in 2010 led to widespread outbreaks [24]. In 2013, PEDV was confirmed for the first time in the United States and quickly spread across the Americas, causing severe economic losses [25]. Today, PEDV is globally distributed enteric pathogen with periodic emergence of novel variants that complicate control efforts.

Virus properties

PEDV is a member of the genus *Alphacoronavirus* in the family *Coronaviridae*. It is an enveloped virus with a positive-sense, single-stranded RNA genome of approximately 28 kb. The genome is organized in the order of 5'-UTR-ORF1a/1b-S-ORF3-E-M-N-3'-UTR. ORF1a/1b encode the replicase polyproteins, processed into non-structural proteins responsible for RNA replication and transcription, while the downstream ORFs encode structural and accessory proteins. The spike (S) glycoprotein mediates receptor binding and membrane fusion, determining tissue tropism, virulence, and antigenicity. ORF3 is linked to viral replication and virulence. Envelope (E) and membrane (M) proteins contribute to virion assembly and budding. Nucleocapsid (N) protein binds viral RNA, forming the ribonucleoprotein complex and modulating host cell responses [26–28]

Although PEDV is less stable than non-enveloped viruses in the environment compared, it can persist for extended periods in organic material (e.g. manure, slurry, feed) and at low temperatures, facilitating indirect oral transmission between farms [29] .

Disease and pathogenesis

PEDV is mainly transmitted via fecal–oral route targeting the mature villous enterocytes in the jejunum and ileum [30]. Infection causes villous atrophy, blunting, and fusion, crypt hyperplasia and intestinal barrier dysfunction [31], leading to malabsorptive diarrhea, reduced digestive capacity, and severe fluid loss. Clinically, PEDV causes sudden onset of profuse, watery diarrhea in pigs of all ages. PEDV causes vomiting, dehydration, and rapid weight loss, especially in neonatal piglets. High morbidity in affected herds and mortality that can approach 100% in piglets less than one week old under severe epizootic conditions [30]. PEDV-infected adult pigs generally show milder signs such as transient diarrhea, anorexia, lethargy, and mild pyrexia, with low mortality [32].

Treatment and prevention

There is no specific antiviral therapy for PEDV. Management focuses on supportive care to reduce mortality and maintain herd productivity. Standard measures include prompt fluid and electrolyte replacement for affected piglets, thermal support, and effective nursing to prevent dehydration and secondary infections [33,34] . At the herd level, control rely on a biosecurity and vaccination. Biosecurity includes quarantine of replacements, all-in/all-out production, and disinfection for environment. Vaccines, including inactivated, live-attenuated, and recombinant types, mainly target the S protein. Maternal immunization enhances lactogenic IgA protection for suckling piglets [35]. However, cross-protection between classical and variant strains is often incomplete, and vaccine efficacy can be variable, highlighting the need for continuous updating of vaccine strains and optimization of immunization protocols.

1.3. Equine coronavirus (ECoV)

History

Coronavirus-associated enteritis in foals was described several decades ago, but the specific role of ECoV was not well defined until the late 1990s and early 2000s. ECoV-infected cases were reported in North America and Europe. In Japan, ECoV emerged as a recognized cause of outbreaks in adult horses at Obihiro Racecourse in Hokkaido, Japan, in 2004, 2009, and 2012. The virus was isolated from diarrheal samples using HRT-18G cells [36–38] .

Virus properties

ECoV is an enveloped, positive-sense single-stranded RNA virus classified within the subgenus *Embecovirus*, species *Betacoronavirus 1*, family *Coronaviridae* [39]. It is closely related to bovine coronavirus and share similar genomic and antigenic features. The genome is approximately 30 kb and encodes the typical coronavirus structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (N), as well as the hemagglutinin-esterase (HE) protein that is characteristic of embecoviruses. The S protein mediates receptor binding and membrane fusion, influencing tropism and host range, while the HE protein facilitates attachment and release from sialylated receptors on host cells. ECoV exhibits genetic flexibility due to its error-prone RNA-dependent RNA polymerase and recombination capacity [40,41]. Although enveloped coronaviruses are generally less stable in the environment than non-enveloped viruses, ECoV can cause enteric infection and persist in feces and bedding under cool conditions, supporting indirect oral- transmission in stabled environments [42].

Disease and pathogenesis

ECoV infection in adult horses typically cause an acute, self-limiting systemic and enteric illness with a 1–4 days incubation. Signs include fever, lethargy, anorexia, and variably soft feces, diarrhea or colic. Leukopenia and neutropenia are frequent, reflecting systemic inflammation [43].

The virus is shed in feces with PCR as the primary diagnostic test [42,44]. Experimental studies on equine intestinal epithelial organoids indicate that ECoV replicates in enterocytes of the small and large intestine, causing cytopathic effects, epithelial damage and disruption of barrier

function, leading to intestinal inflammation, fluid imbalance and malabsorptive diarrhea [45]. Most horses recover with supportive care, but severe complications such as necrotizing enteritis, endotoxemia and hyperammonaemia encephalopathy can occur, particularly in miniature horses. Neurologic signs including ataxia, head pressing and seizures may arise secondary to hyperammonemia and systemic inflammation, occasionally resulting in death [46,47].

Treatment and prevention

No ECoV-specific antiviral therapy or licensed vaccine exist to date. Current management focused on supportive treatment and strict biosecurity. Prevention relies on prompt isolation and testing of symptomatic horses, use of dedicated equipment, clothing and personnel for sick horses, careful manure handling through cleaning and disinfection of stalls, and movement restrictions during outbreaks [48]. In the absence of a vaccine, consistent implementation of these measures remains the primary defence. Ongoing research into ECoV genomics and host–virus interactions may eventually lead to effective vaccines or targeted therapies.

1.4. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

History

SARS-CoV-2, the virus responsible for COVID-19, was first identified in Wuhan, China, in December 2019 [49]. Its rapid human-to-human transmission led to a global. Several variants of concern (VOCs) including Alpha, Delta, and Omicron, have since emerged, driven by spike protein mutations enhancing transmissibility and immune evasion [50]. Widespread vaccination and prior infections have shifted the global response from acute crisis to endemic management, yet SARS-CoV-2 remains a major respiratory pathogen [51]. Severe outcomes are most common in older adults, those with underlying conditions, and the immunocompromised [52]. Infections in animals raise concerns about wildlife reservoirs and potential zoonotic spillback [53,54].

Virus properties

SARS-CoV-2 is an enveloped, positive-sense single-stranded RNA virus of the *Betacoronavirus* genus (subgenus *Sarbecovirus*), with approximately 30 kb genome encoding non-structural proteins for replication and transcription, along with structural and accessory proteins. Key structural components include the spike (S) protein, which binds ACE2 via its S1 subunit and mediates membrane fusion through S2, driving tropism, transmission, and antigenicity. The membrane (M) and envelope (E) proteins, essential for virion assembly. The nucleocapsid (N) protein, which packages the genome and modulates host responses. The virus is ~80–120 nm in diameter with prominent surface spikes. Its error-prone RNA polymerase and recombination ability support rapid evolution and emergence of immune-evasive variants [55].

Disease and pathogenesis

COVID-19 presents with a wide clinical spectrum, ranging from asymptomatic cases to critical illness. Common acute manifestations include fever, cough, sore throat, nasal congestion, fatigue, myalgia, and loss of smell or taste, with many also experiencing gastrointestinal symptoms. Severe disease, more common in older adults and those with comorbidities, may progress to viral pneumonia, hypoxemia, and acute respiratory distress syndrome (ARDS). Pathologically, severe

COVID-19 features diffuse alveolar damage, endothelial injury, and widespread microthrombi, driven by both viral cytopathic effects and immune dysregulation. Multisystem involvement is common, with complications such as myocarditis, arrhythmias, thromboembolism, acute kidney injury, and encephalopathy [56–58] . Additionally, a subset of patients develops long COVID, characterized by persistent fatigue, dyspnea, and cognitive impairment, with underlying mechanisms still under investigation [58].

Treatment and prevention

While there is no definitive therapy for COVID-19, treatment now combines antivirals, immunomodulators, and supportive care tailored to disease severity and patient risk. Prevention relies primarily on vaccination and public health measures. mRNA, adenoviral-vectored, and protein-based vaccines offer strong protection against severe outcomes, despite waning effectiveness against infection. Updated boosters targeting Omicron subvariants are advised for high-risk groups. Complementary strategies include enhanced ventilation, high-filtration masks, isolation when symptomatic, targeted testing, and protecting vulnerable healthcare settings [59] .

1.5. Influenza A (H1N1) (FluV)

History

The 1918–1919 “Spanish flu” pandemic, caused by an avian-origin H1N1 influenza A virus, infected an estimated one-third of the global population and led to at least 50 million deaths, making it the deadliest influenza outbreak of the 20th century [60]. Genetic studies of archival samples have confirmed that the 1918 virus was an H1N1 strain and have provided key insights into its unusual virulence [61]. After 1918, H1N1 circulated seasonally until replaced by H2N2 in 1957, then re-emerged in 1977 alongside H3N2 [62]. In 2009, a novel swine-origin H1N1 virus (A(H1N1)pdm09) emerged in North America, triggering a global pandemic. The virus was a triple-reassorting swine influenza strain containing gene segments from avian, human and swine lineages. It caused over 60 million U.S. cases and 12,500 deaths in its first year [63]. Since 2010, this virus has become established as a regular seasonal H1N1 lineage, replacing the pre-2009 human H1N1 strains [64].

Virus properties

Influenza A H1N1 viruses are enveloped, pleomorphic particles with surface glycoproteins hemagglutinin (H1) and neuraminidase (N1) embedded in a host-derived lipid membrane. Their genome comprises eight single-stranded, negative-sense RNA segments encoding at least 17 proteins, including polymerase subunits (PB2, PB1, PA), HA, NA, NP, M1, M2, and NS proteins. Each segment forms a ribonucleoprotein complex with NP and the RNA-dependent RNA polymerase, and virions must selectively incorporate one copy of each segment to be infectious. This segmented structure allows antigenic shift through reassortment during co-infection with different influenza A strains, enabling the emergence of pandemic viruses like A(H1N1)pdm09. Concurrently, the virus evolves via antigenic drift—gradual mutations in HA and NA—driving seasonal variation and vaccine escape [65].

Disease and pathogenesis

Seasonal A(H1N1) influenza presents similarly to other influenza A subtypes, with abrupt onset of fever, chills, myalgia, headache, malaise, dry cough, sore throat, and nasal congestion after a

1–4 day incubation [66]. Children often experience higher fevers and gastrointestinal symptoms. The virus primarily replicates in the respiratory epithelium, from the upper airways to the lower tract in severe cases [67]. While typically self-limiting in healthy individuals, high-risk groups may develop viral pneumonia, ARDS, or secondary bacterial infections. Severe cases often involve diffuse alveolar damage and intense inflammation. The 2009 pandemic saw disproportionate severity in young adults and pregnant women, reflecting differences in immunity and host response. Less common complications include myocarditis, encephalopathy, and multi-organ failure, while influenza can also exacerbate chronic conditions and trigger cardiovascular events [68] .

Treatment and prevention

Adjunctive measures include respiratory hygiene (mask use in high-risk settings, cough etiquette), staying home when ill, improved ventilation, and, during outbreaks in closed settings, post-exposure antiviral prophylaxis for close contacts at increased risk of complications. While there is no virus-specific cure for A(H1N1), early antiviral treatment, vaccination, and public health measures significantly reduce morbidity and mortality. Recommended antivirals include neuraminidase inhibitors (oseltamivir, zanamivir, peramivir) and the cap-dependent endonuclease inhibitor baloxavir marboxil, particularly for hospitalized patients, those with severe or progressive illness, or high-risk individuals. Treatment is most effective within 48 hours of symptom onset, though later use may still benefit severe cases [66]. Annual vaccination remains the primary strategy for prevention. Seasonal influenza vaccines, updated based on global surveillance, include A(H1N1)pdm09 and other circulating strains, offering strong protection against severe outcomes, especially in vulnerable groups [69] . Complementary measures include mask use in high-risk settings, respiratory hygiene, staying home when ill, improving indoor ventilation, and antiviral prophylaxis during outbreaks in high-risk environments [70].

Non-enveloped viruses

1.6. Human adenovirus D56 (HAdV-D56)

History

Human Adenovirus genotype D56 (HAdV-D56) was discovered in France in 2008 from a fatal case of neonatal infection [71]. In 2011, Robinson et al. [72] reported the computational and genomic analysis of this antigen as a novel recombinant species D adenovirus, HAdV-D56. In 2012, the first documented case of male urethritis due to HAdV-56, was identified in Japan by PCR and sequencing [73]. In 2014, epidemic keratoconjunctivitis (EKC) outbreak caused by HAdV-D56 in Dalian, China in 2012 [74]. In 2021, fatal neonatal sepsis cases were reported in the United States caused by HAdV-D56-like strains during 2014-2020 [71]. More recently, molecular epidemiology surveys have identified HAdV-D56 among pediatric patients with respiratory disease and acute gastroenteritis in Japan, suggesting that its clinical spectrum and transmission routes may be broader than initially appreciated [75].

Virus properties

Adenoviruses are non-enveloped, icosahedral, double-stranded DNA viruses of approximately 26-45kb in length. The virus capsid is composed mainly of hexon, penton base and fiber proteins, which determine cell attachment and tissue tropism. Human adenovirus (HAdV) is currently classified by phylogenomics into seven species (A-G). Human Adenovirus species D is primary causative agent of EKC, including HAdV-D8, 37, 53, 54, 56. HAdV-D56 is a natural recombinant adenovirus with a novel penton base hypervariable region (HVR1), the penton base HVR2 of HAdV-D9, the hexon gene of HAdV-D15, and the fiber gene of HAdV-D9. Owing to their protein capsid, adenoviruses show persistence on hands, fomites, and even metal and plastic surfaces [76–79]. Recent studies have reported that HAdV-D56 uses CD46 as a major cellular receptor through a noncanonical interaction with the hexon protein rather than the fiber knob [80,81].

Disease and pathogenesis

HAdVD-56 has been reported to cause EKC and urethritis in adult human, sepsis or pneumonia in neonates, and respiratory or gastrointestinal disease in children, depending on host tropism and immune responses. EKC typically presents as follicular conjunctivitis (pinkeye), with a pebbled or nodular appearance of the palpebral conjunctival mucosa and inflammation of both conjunctivae [79]. Neonatal systemic infection is associated with diffuse viral pneumonia and viremia with multiorgan involvement [71] . An In vivo study demonstrated that HAdV-D56 targets the spleen and lungs in mice, induce cellular immune responses with robust cytokine (IFN γ , TNF α , IL5, IL6, IP10, MCP1 and MIG1) production leading to severe inflammation, underscoring its potential for strong innate immune activation in patients [82] .

Treatment and prevention

Currently no antiviral drug or licensed vaccine is available. Management of HAdV-D56 focuses on supportive care on clinical symptoms. Prevention is therefore critical and relies on hand hygiene, avoidance of sharing personal items and thorough environmental disinfection with effective agents against adenovirus [83,84].

1.7. Feline calicivirus (FCV)

History

FCV was first identified in the late 1950s, with the classic vaccine strain FCV-F9 isolated around 1950–1957 and later adopted as a prototype for experimental work and vaccine development [85]. FCV is prevalent in both domestic and wild felids, with infection rates around 10% in household cats and 25–40% in shelters or catteries, reflecting differences in population density and management [86,87]. Transmission occurs primarily through direct contact with oronasal secretions, but also via feces, urine, fomites, and aerosols, especially in confined spaces. Many cats recover clinically but become chronic or intermittent carriers, shedding virus over months to years and seeding new infections, especially in multi-cat environments [88].

Virus properties

FCV is a small, non-enveloped virus with icosahedral symmetry and characteristic cup-shaped surface depressions. Its approximately 7.5 kb positive-sense RNA genome contains three open reading frames: ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase; ORF2 and ORF3 encodes VP1 and VP2 capsid proteins [89]. FCV's environmental resilience and resistance to some disinfectants contribute to its persistence in cat colony [88]. FCV are frequently used as surrogate virus for human norovirus [90].

Disease and pathogenesis

FCV primarily targets epithelial cells of the oropharynx and upper respiratory tract, but some strains can also affect the lower airways, oral mucosa, joints, and multiple organs in systemic disease. Clinical signs include acute upper respiratory symptoms, oral ulcers with salivation and sometimes dysphagia, chronic gingivostomatitis linked to persistent infection, and a transient polyarthritis syndrome [85]. Virulent systemic FCV (VS-FCV) causes severe illness with high fever, edema, skin ulceration, jaundice, and multi-organ failure, with outbreak mortality rates exceeding 22–86% [85,91]. These cases involve widespread endothelial damage, vasculitis, and dysregulated inflammation. [85]

Treatment and prevention

No FCV-specific antiviral drug is used in routine clinical use. Management focuses on supportive care and transmission control. Treatment includes fluid therapy, nutritional support, analgesia, and management of oral pain and ulcers [92]. Broad-spectrum antibiotics may be used for suspected secondary infections, while chronic gingivostomatitis may require dental extractions or immunomodulatory therapies. Vaccination remains central to prevention, with live-attenuated or inactivated vaccines commonly combined with feline herpesvirus-1 and panleukopenia virus. Although vaccination reduces disease severity, it does not provide sterilizing immunity and may offer limited protection against divergent strains [86,93]. Effective biosecurity including strict hygiene, disinfection, isolation, and proper shelter management is critical to limit viral spread [93] .

1.8. Coxsackie virus B2 (CVB2)

History

Coxsackie virus B2 (CVB2) were first isolated in 1948 from paralyzed children in Coxsackie, New York, and later classified into group A and B based on pathogenicity in mice and antigenic traits. Group B viruses, including CVB2, were quickly associated with myocarditis, pleurodynia, and pericarditis, especially in children and young adults [94]. CVB2 has circulated worldwide but has mainly caused focal outbreaks rather than nationwide epidemics. In 1989, Alexander et al. reported cluster infections in high school in United States [95]. In 2022, a cluster of meningoencephalitis cases were reported in Israel [96]. Overall, CVB2 tends to appear as regional or institutional outbreaks of CNS or systemic disease during peak enterovirus seasons.

Virus properties

CVB2 is a small (~30 nm), non-enveloped, icosahedral virus in the genus *Enterovirus*, composed of a VP1–VP4 capsid enclosing a ~7.4 kb positive-sense single-stranded RNA genome. The genome includes a 5' untranslated region (UTR) with an internal ribosome entry site (IRES), a single open reading frame encoding a polyprotein cleaved into structural (VP1–VP4) and non-structural (2A–2C, 3A–3D) proteins, and a 3' UTR with a poly(A) tail. CVB2 exhibits high mutation rates due to its error-prone RNA polymerase and undergoes frequent recombination within the *Enterovirus B* species, generating substantial genetic diversity, which contributes to variation in virulence, tropism, and immune evasion [94].

Disease and pathogenesis

CVB2 causes a broad spectrum of disease, including nonspecific febrile illness, respiratory symptoms, aseptic meningitis, encephalitis, myocarditis, pericarditis, pleurodynia, pancreatitis, neonatal sepsis-like illness, and occasionally hand-foot-mouth disease or acute flaccid paralysis [97]. Neonates are particularly vulnerable, presenting with multi-organ involvement including hepatitis, myocarditis, and CNS disease. Pathogenesis involves initial replication in the oropharynx and gut,

followed by viremia and dissemination to target organs, with tissue injury driven by direct viral cytopathy, innate immunity, and possible autoimmune responses [98].

Treatment and prevention

There is no licensed antiviral therapy for CVB2 or most enteroviruses. Management is supportive and based on disease severity and organ involvement. Treatment includes antipyretics and analgesics, fluid and nutritional support (especially in infants), cardiac monitoring and standard heart failure care for myocarditis, and intensive care for severe CNS or neonatal sepsis-like presentations. While capsid-targeting antivirals like pleconaril show promise, they are not routinely available [99].

Prevention focuses on hygiene and transmission control, including handwashing, surface disinfection, and avoiding contact with symptomatic individuals. No CVB2 vaccine is currently licensed, but experimental vaccines targeting CVB antigens are in development, especially for myocarditis and type 1 diabetes prevention [97].

1.9. Microbial detergent for various virus

Detergents and disinfectants used in the outer environment are usually formulated to be *strongly microbicidal* and are not intended for direct contact with tissues. Acidic detergents combine surfactants with low pH such as citric acid, acetic acid, oxalic acid to remove organic and inorganic deposits such as scale, proteins and biofilms from surfaces. The acidic environment itself can destabilize some viruses and bacteria, and the surfactant component helps to solubilize lipids and disrupt microbial envelopes [100–102].

In addition, agents used on human body surfaces such as eyes and skin must balance antimicrobial activity with biocompatibility. Sodium hypochlorite and hydrogen peroxide are oxidizing agents that damage microbial membranes, proteins and nucleic acids [103]. By carefully controlling formulations, they are used for wound irrigation, contact lens disinfection or ocular surface cleaning. Salicylic acid has keratolytic and mild antimicrobial effects and is widely used on skin and used for contact lens disinfection [104]. Polyhexamethylene biguanide (PHMB), ϵ -polylysine, polyquaternium-1 (PQ-1) and alexidine are cationic antiseptics, binding to negatively charged microbial membranes, increase permeability and lead to leakage and death, while being tolerated by human tissues at low concentrations [105–107]. Many commercial eyes drop and contact lens solutions therefore combine surfactant action with one or more of these cationic biocides, providing sustained antimicrobial protection on the ocular surface without the tissue damage.

1.10. Human gastrointestinal acid as acidic disinfectant inside human body

Human gastric acid forms a primary microbiocidal barrier to prevent antigen to reach into the intestine [108,109]. Gastric juice is a mixture of hydrochloric acid (HCl), pepsin and other components, with a fasting pH typically around 1–2 and increase to 6 after food ingestion [110]. The prevailing assumption suggested that many enveloped viruses lose infectivity at low pH. However, the effectiveness of this virucidal barrier in gastric lumen depends on the antigen characteristic. Even so, food buffering in gastric lumen after meal could largely change pH environment and pepsin activity to allow antigen to sneak into intestine.

1.11. Unknown facts

Most commonly used detergents and disinfectants have been characterized primarily in terms of their antibacterial spectrum, minimum inhibitory concentrations, and material compatibility, whereas their activity against viruses has been much less systematically studied. In routine practice, it is often assumed that enveloped viruses are sensitive to low pH environment due to its enveloped structure, while non-enveloped viruses are more resistant due to its protein capsids.

However, this paradigm is largely based on general principles and limited experimental datasets for virucidal assessment. In reality, virucidal efficacy is likely to depend not only on the presence or absence of an envelope, but also on the detailed physicochemical properties of individual viruses and the specific formulation, concentration, pH, and contact time of each detergent.

To address these knowledge gaps, we systematically evaluated the virucidal effects of a panel of detergents with different chemistries and human gastric acid. By testing enveloped and non-enveloped viruses under controlled conditions, our study provides a more evidence-based framework for interpreting detergent efficacy, for selecting appropriate agents in environmental and surface decontamination. In addition, we analyse coronavirus infectivity in human gastric acidity to provide scientific support for coronavirus maintaining its infectivity inside human gastric acid.

2. CHAPTER 1

**pH-dependent virucidal effects of weak acids
against pathogenic viruses**

2.1. Abstract

Background: Weak acids, such as acetic acid, show virucidal effects against viruses, and disinfectants are considered effective virucidal agents possibly because of their low pH, depending on the proton concentration. This study aimed to evaluate the efficacy of different weak acids (acetic, oxalic, and citric acids) and eligible vinegars under different pH conditions by comparing their inactivation efficacy against enveloped and non-enveloped viruses.

Methods: Acetic, oxalic, and citric acids were adjusted to pH values of 2, 4 and 6, respectively. They were also diluted from 1 M to 0.001 M with distilled water. Enveloped influenza A virus (FulV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and non-enveloped feline calicivirus (FCV) were treated with adjusted weak acids for up to 30 min. These viruses were also reacted with white distilled vinegar (WDV) and grain-favoured distilled vinegar (GV) for up to 30 min. Infectious viral titers after the reactions were expressed as plaque-forming units per mL.

Results: Acetic acid showed virucidal effects against FulV at pH 4, whereas citric and oxalic acids did not. Acetic and citric acids inactivated SARS-CoV-2 at pH 2, whereas oxalic acid did not. All acids showed virucidal effects against FVC at pH 2; however, not at pH 4. The virucidal effects of the serially diluted weak acids were also reflected in the pH-dependent results. WDV and GV significantly reduced FulV titers after 1 min. SARS-CoV-2 was also susceptible to the virucidal effects of WDV and GV; however, the incubation period was extended to 30 min. In contrast, WDV and GV did not significantly inactivate FCV.

Conclusions: The inactivation efficacy of weak acids is different even under the same pH conditions, suggesting that the virucidal effect of weak acids is not simply determined by pH, but that additional factors may also influence these effects. Moreover, eligible vinegars, the main component of which is acetic acid, may be potential sanitizers for some enveloped viruses, such as FulV, in the domestic environment

2.2. Introduction

Viral infectious diseases, such as coronavirus disease 2019 [111–113], influenza [114–116] and norovirus gastroenteritis [117,118] are major domestic public health concerns. These diseases are highly contagious, which makes the inactivation of these viruses a matter of utmost priority in preventing the spread of infections. Inactivation of these viruses is of the utmost priority in preventing the spread of infections.

Disinfectants, such as ethanol [119,120] and sodium hypochlorite [121,122] are generally used as potent virucidal agents domestically. Weak acids, such as acetic acid, are also effective against pathogenic viruses. Acetic acid exerts virucidal effects against enveloped viruses, such as vaccinia virus, African swine fever virus, influenza virus (FulV), and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [123–127]. In addition, vinegar, the main component of which is acetic acid, shows efficient virucidal activity against SARS-CoV-2 [125,128,129]. It is a natural product that is harmless to the human body [130–132]; thus, the use of edible vinegars as cleaning and sanitizing agents is promising domestically [133–135].

The virucidal effect of acids is considered to be due to the low pH. Therefore, any weak acid seems to exert similar inactivation effects under the same pH conditions. However, the effective pH of weak acids has not yet been fully elucidated. In this study, we aimed to compare the inactivation effects of acetic, oxalic, and citric acids against two enveloped viruses, SARS-CoV-2 and FulV, and a non-enveloped virus, feline calicivirus (FCV), under different pH conditions. We further demonstrated the virucidal effects of eligible vinegars. These observations provide useful information regarding the appropriate conditions for weak acids against pathogenic viruses.

2.3. Materials and methods

2.3.1 Viruses and cells

SARS-CoV-2 (2019-nCoV/Japan/AI/I-004/2020, National Institute of Infectious Diseases [NIID] strain) was kindly provided by the NIID (Tokyo, Japan). FulV A/H1N1 was provided by the Yamaguchi Prefecture Institute of Public Health and Environment (Yamaguchi, Japan). FCV was obtained from the American Type Culture Collection and was used as a surrogate for norovirus.

FulV, SARS-CoV-2, and FCV were propagated in Madin–Darby canine kidney (MDCK), VeroE6/TMPRSS2 (JCRB 1819), and Crandell–Rees Feline Kidney (CRFK) cells, respectively. VeroE6/TMPRSS2 was kindly provided by the NIID. These cells were propagated in Dulbecco's modified Eagle's minimum essential medium (DMEM; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (JR Scientific, Woodland, CA, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Carlsbad, CA, USA) and maintained at 37 °C in 5 % CO₂.

Virus-infected cells were incubated at 37 °C in 5 % CO₂ in DMEM supplemented with 2 % FBS. The supernatants of these growth media were stored at –80 °C as stock sources of viruses. Stocked virus titers of FulV, SARS-CoV-2 and FCV were 6.33×10^6 plaque-forming assay units (pfu)/ml, 6.93×10^7 pfu/ml and 1.04×10^7 pfu/ml, respectively.

Experiments using infectious SARS-CoV-2 were performed in a biosafety level (BSL)-3 laboratory at Yamaguchi University following standard BSL-3 guidelines. FulV and FCV experiments were performed in a BSL-2 laboratory.

2.3.2 Plaque-forming assay

The infectious viral titers of SARS-CoV-2, FulV, and FCV were determined using a plaque-forming assay. Briefly, each type of cell was grown in 12-well plates until confluent; subsequently, serially diluted viruses were inoculated (100 µL/well). SARS-CoV-2 and FCV were diluted in DMEM supplemented with 2 % FBS, and FulV was diluted in DMEM supplemented with 0.001 % trypsin (trypsin from Hog pancreas 1:250; NACALAI TESQUE, Tokyo, Japan) and 0.2 % bovine serum albumin (BSA) (SIGMA, USA). After incubation at 37 °C for 90 min, the inoculum was removed,

and the cells were washed with DMEM. Subsequently, 0.8 % agarose (SeaPlaque GTG Agarose, Lonza, Rockland, ME, USA) in DMEM supplemented with 10 % FBS or DMEM supplemented with 0.001 % trypsin and 0.2 % BSA was added to each well. The plates were incubated at 37 °C until visible plaques appeared. Plaque formation was observed at 36 h post-infection (pi) for SARS-CoV-2, 48 h pi for FulV, and 24 h pi for FCV. Addition of trypsin to media did not influence pH of reaction samples for FulV including acids and plaque formation (data not shown). To retrieve the plate, the cells were fixed with 10 % formalin and thoroughly stained with 1 % crystal violet (FUJIFILM WAKO, Osaka, Japan). The viral titers were expressed as plaque-forming units (pfu)/mL.

2.3.3 Weak acids

In total, 1 M acetic acid (FUJIFILM, conduct log: 017-00256), 1 M oxalic acid (WAKO, conduct log: 150-00455), and 1 M citric acid (FUJIFILM, conduct log: 038-06925) were diluted using sterilized water from the original acid solutions. To adjust the acetic acid to pH 2, 4, and 6, 0.12-, 0.2-, and 1-time volumes of 1 M sodium hydroxide (NaOH) were added to 1 M acetic acid, respectively. Correspondingly, 1.08-, 1.5-, and 1.9-time volumes of 1 M NaOH and 0.14-, 1.3-, and 2.59-time volumes of 1 M NaOH were added to 1 M oxalic acid and 1 M citric acid, respectively, to adjust the pH to 2, 4, and 6. Each 1 M acid was serially diluted to 1, 0.1, 0.01, and 0.001 M with sterilized water.

2.3.4 Inactivation of viruses by weak acids

A total of 900 µL of each adjusted weak acid was mixed with 100 µL of each viral solution in 2 % FBS-supplemented DMEM at a 9:1 ratio and reacted for 1-30 min at room temperature. The pH of the reaction mixtures did not significantly change from the original adjusted values (Table 1).

The reacted solutions were immediately diluted with 2 % FBS-supplemented DMEM, and viral titers were determined. Notably, pH values and of the weak acid- and edible vinegar-reacted solutions for plaque assays were confirmed (Table S1, S2 and S3). Cell viabilities of each cell for plaque assay were also evaluated using XTT cell viability assay kit (Biotium, conduct log: 30007, CA, USA) (Table S4 and S5). From these data, more than 1:100 dilution of any acids didn't induce cell damages, indicating that the detection limit of viral titers was 10^3 pfu/ml for each virus. The effectiveness of the weak acids was determined based on viral loads below the detection limit.

2.3.5 Vinegars

White distilled vinegar (WDV) and grain-flavored distilled vinegar (GV) containing 4 % acetic acid were provided by Mizkan Holdings Co., Ltd., Aichi, Japan. In total, 100 μ L of each viral solution was mixed with 900 μ L of WDV, GV, and 4 % acetic acid for 1, 5, and 30 min, respectively, at room temperature. Subsequently, the samples were immediately diluted with DMEM containing 2 % FBS and were applied to cultured cells for titration using the plaque-forming assay. The effectiveness of the weak acids was determined based on viral loads below the detection limit. Notably, > 1:100 dilutions did not cause any cell damage, indicating that the detection limit of the virus titer was 10^3 pfu/mL.

2.4. Results

2.4.1. pH-dependent virucidal effects of weak acids

Against FulV, acetic acid caused significant reductions in viral titers below the detection limit at pH 2 and 4 in 1 min; however, not at pH 6 (Fig. 1a). In contrast, citric and oxalic acids substantially reduced the titers at pH 2 in 1 min; however, it did not show virucidal effects at pH 4 (Fig. 1a) in 1 min. Acetic acid at pH 4 significantly reduced FulV titer under detection limit within 1 min, while oxalic and citric acid required 10 min to reduce viral titration. At pH 6 of weak acids, viral titer mildly reduced in 30 min while compared to 1 min. These observations suggest that acetic acid shows potentially stronger effects against FulV than oxalic and citric acids under the same pH conditions.

Interestingly, the effects of weak acids on SARS-CoV-2 were different from those on FulV, although both are enveloped viruses. Acetic and citric acids caused a significant reduction in viral titers of SARS-CoV-2 at pH 2 in 1 min, whereas oxalic acid did not (Fig. 1b). None of the acids exhibited a significant reduction in viral titers at pH 4 and 6 (Fig. 1b). Although viral titers were somewhat reduced after 30 min incubation in each reaction, the inactivation effect of oxalic acid were comparatively limited than acetic and citric acids (Fig 1). These results indicate that the virucidal effect of oxalic acid is limited to SARS-CoV-2 when compared with that of acetic and oxalic acids under low pH (pH 2) conditions.

Acetic, oxalic, and citric acids significantly reduced virus titers of FCV to below the detection limit at pH 2 in 1 min; however, the same reduction was not observed at pH 4 and 6 (Fig. 1c), indicating that the inactivation effects against FCV are not significantly different among acetic, citric, and oxalic acids.

These findings suggest that the inactivation efficacy of weak acids differs for different viruses under the same pH conditions. Acetic acid has a potentially greater effect compared with citric acid and oxalic acids.

2.4.2. Concentration-dependent virucidal effects of weak acids

The virucidal effects of each acid, depending on serial concentrations of 0.001–1 M, were also

examined (Table 2). In total, 0.01–1 M acetic acid (pH 2.39–4.46), citric acid (pH 1.27–2.94), and oxalic acid (pH 0.63–2.25) showed significant reductions of FulV titers (Fig. 2a), whereas 0.001 M acetic acid (pH 7.35), citric acid (pH 6.41), and oxalic acid (pH 6.79) did not. These results corresponded to the pH-dependent effects shown in Fig. 1a.

Against SARS-CoV-2, 1 M acetic acid (pH 2.39) showed a significant reduction in viral titers; however, 0.001–0.1 M (pH 3.25–7.35) did not (Fig. 2b). Moreover, 1 M (pH 1.27) and 0.1 M (pH 2.02) citric acid exhibited significant inactivation of viral titers; however, 0.01 M (pH 2.94) and 0.001 M (pH 6.41) did not (Fig. 2b). Additionally, 1 M (pH 0.63) and 0.1 M (pH 1.20) oxalic acid showed significant reduction of viral titers; however, 0.01 M (pH 2.25) and 0.001 M (pH 6.79) did not (Fig. 2b). These effects corresponded to the pH-dependent results shown in Fig. 1b.

Against FCV, 1–0.001 M acetic acid (pH 2.39–7.35) did not show significant inactivation (Fig. 2c). Moreover, 1 M (pH 1.27) citric acid showed virucidal activity; however, 0.1–0.001 M citric acid (pH 2.02–6.41) did not (Fig. 2c). Furthermore, 1 M (pH 0.63) and 0.1 M (pH 1.20) oxalic acid significantly inactivated FCV; however, 0.01 M (pH 2.25) and 0.001 M (pH 6.79) did not (Fig. 2c). These results mostly corresponded to the pH-dependent results shown in Fig. 1c, although the effects of approximately pH 2, including 0.1 M citric acid (pH 2.02) and 0.01 M oxalic acid (pH 2.25), were different from the results of Fig. 1c. These differences may be due to the dependence of the efficacy on the acid concentration.

These observations suggest that pH mainly influences the virucidal activity of each weak acid. Taken together, acetic acid can inactivate FulV at approximately pH 4; however, citric and oxalic acids require pH lower than 4 for the same efficacy. In addition, acetic and citric acids can inactivate SARS-CoV-2 at approximately pH 2, whereas oxalic acid requires a pH < 2. These findings suggest that the virucidal activity of weak acids does not simply depend on proton concentration, and other factors of each weak acid may also contribute significantly to these effects.

2.4.3. Virucidal effects of eligible vinegars

Our observations imply that acetic acid effectively reduces the infectious virus concentrations under higher pH conditions compared with other weak acids. Therefore, we examined the virucidal activity of vinegars containing acetic acid as the main component.

Similar to 4 % acetic acid (pH 2.72), 4 % WDV (pH 2.77) and 4 % GV (pH 2.79) exhibited significant reductions in FulV titers below the detection limit immediately after 1 min (Fig. 3a). These results reflect the pH-dependent effects of acetic acid on the reaction solutions.

SARS-CoV-2 was also susceptible to the virucidal effects of 4 % WDV and 4 % acetic acid; however, incubation periods below the detection limits were extended up to 30 min (Fig. 3b). Moreover, 4 % GV did not reach the detection limit after 30 min (Fig. 3b). These observations also reflect the pH-dependent effects of acetic acid.

In contrast, 4 % WDV (pH 2.77), 4 % GV (pH 2.79), and 4 % acetic acid (pH 2.72) did not significantly inactivate FCV (Fig. 3c). These results are consistent with those of the adjusted 1 M acetic acid (pH 2.51) shown in Fig. 2c.

These observations suggest that eligible vinegars may be potential sanitizers for some enveloped viruses, such as FulV, in the domestic environment; however, these effects may be limited to enveloped SARS-CoV-2 and non-enveloped viruses.

2.5. Discussion

Generally, virucidal activity of acids is due to their low pH, which depends on the molar concentration of protons. Therefore, a higher molar proton concentration is believed to be the key determinant, regardless of the type of acid. Interestingly, our results suggest that there are cases in which inactivation efficacy varies between different types of weak acids, even under the same pH conditions. For example, acetic acid at pH 4 inactivated FulV, whereas citric and oxalic acids did not. Moreover, pH 2 acetic and citric acids showed significant virucidal effects against SARS-CoV-2, whereas oxalic acid did not. These observations suggest that the virucidal effect of weak acids is not simply determined by the pH, and additional factors, such as chemical structure of the acid and other factors, may influence these effects. For example, citric acid and oxalic acid are dicarboxylic acid, and this chemical structure may influence the different effects compared with acetic acid. Further studies will be required to identify the mechanism of inactivation effects due to different weak acids. In addition, underlying the morphological changes of inactivated viruses using cryo-electron microscope will provide useful information for understanding the inactivation mechanism by weak acids.

Our results showed that acetic acid was more effective than oxalic and citric acids against the enveloped viruses FulV and SARS-CoV-2 at higher pH. Interestingly, acetic acid induces unique physiological responses in mammalian cells when compared with other types of acids [136], suggesting that acetic acid may penetrate the lipid bilayer of the cell membrane. Therefore, acetic acid may cause physiological changes in the viral envelope and is a potentially effective disinfectant.

Acetic acid is the primary component of vinegar. Edible vinegar is produced by fermentation of plant-based products and is harmless to the human body [131]. It is generally used as a condiment domestically. Vinegar has virucidal effects against SARS-CoV-2 [128]. In this study, 4 % WDV and 4 % GV showed virucidal efficacy against SARS-CoV-2 and higher effectiveness against FulV. However, these inactivation effects were limited against FCV. 4 % GV showed less potency of virucidal effect against SARS-CoV-2 compared with 4% WDV and 4% AA, although they should contain same kind and concentration of weak acid with similar pH. GV and WDV contain other organic components extracted from plants such as sugar, and these different components may

influence the effects of GV and WDV. Our results demonstrate the potential antiviral properties of these edible vinegars, which can be used as sanitizers against enveloped viruses in common household.

Interestingly, our results showed that SARS-CoV-2 was comparatively stable against inactivation by acetic and oxalic acids under the same pH conditions when compared with FulV, although both viruses are enveloped. In particular, oxalic acid did not inactivate SARS-CoV-2, even at pH 2. These observations indicate that this virus is more stable under low pH conditions than other enveloped viruses. A previous study showed that SARS-CoV-2 was extremely stable over a wide pH range [137]. Wang et al. [138] recently showed that oxalic acid exhibited inhibitory effects on combination to the receptor-binding domains of the SARS-CoV-2 pseudovirus and angiotensin-converting enzyme 2. Although our data showed no changes in infectious ability of SARS-CoV-2 after oxalic acid reactions, the elucidation of the inconsistent observations may provide evidence to reveal the factors affecting the stability of SARS-CoV-2 under acidic conditions.

Some coronaviruses, such as transmissible gastroenteritis virus [139] and porcine epidemic diarrhea virus [140], cause oral infections and intestinal diseases, such as diarrhea, suggesting that these coronaviruses are resistant to gastric acid. Therefore, coronaviruses potentially possess resistance to low pH among enveloped viruses. It would be interesting to reveal the mechanism and factors underlying the low pH stability of enveloped coronaviruses, which cause gastrointestinal infections. Further investigation of these factors will provide useful information for the development of more effective antiviral agents against coronaviruses, including SARS-CoV-2.

2.6. Conclusion

When comparing acetic, oxalic, and citric acids, we observed that the inactivation efficacies of weak acids are different, even under the same pH conditions. These observations suggest that the virucidal effect of weak acids is not simply determined by pH and additional factors may also influence these effects. Acetic acid shows an effective virucidal reaction against enveloped viruses compared with oxalic and citric acids. Eligible vinegars, whose main component is acetic acid, may be potential sanitizers for some enveloped viruses, such as FulV in the domestic environment. In addition, our data imply that SARS-CoV-2 is comparatively stable against inactivation by weak acids. Further investigation to reveal the mechanism and factors of low pH stability of enveloped coronaviruses will provide useful information for the development of more effective antiviral agents against coronaviruses.

2.7. Figure Legends

Fig. 1. pH-dependent virucidal effects of acetate, oxalate, and citrate against influenza A virus (FulV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and feline calicivirus (FCV).

Acetate, oxalate, and citrate adjusted at pH 2, 4, and 6 were mixed with each virus for 1, 10 and 30 min. Negative control (NC) was incubated with distilled water instead of weak acids. Plaque-forming unit (pfu) per mL of FulV (A), SARS-CoV-2 (B), and FCV (C) were determined. Error bars indicate the standard error. Detection limit was set to 10^3 pfu/mL. Each viral inactivation was accessed in triplicate.

Fig. 2. Concentration-dependent virucidal effects of acetate, oxalate, and citrate against FulV, SARS-CoV-2 and FCV. In total, 1, 0.1, 0.01, and 0.001 M of acetate, oxalate, and citrate were mixed with each virus for 1 min. NC was incubated with distilled water instead of weak acids. pfu per mL of FulV (a), SARS-CoV-2 (b), and FCV (c) were determined. Error bars indicate the standard error. Detection limit was set to 10^3 pfu/mL. Each viral inactivation was accessed in triplicate.

Fig. 3. Virucidal effects of eligible vinegars. In total, 4 % white distilled vinegar (WDV), 4 % grain-flavored distilled vinegar (GV), and 4 % acetic acid (AA) were mixed with each virus for 0 (control), 1, 5, and 30 min. pfu per mL of FulV (a), SARS-CoV-2 (b), and FCV (c) were determined. Error bars indicate the standard error. Detection limit was set to 10^3 pfu/mL. Each viral inactivation was accessed in triplicate.

2.8. Figures

Fig. 1

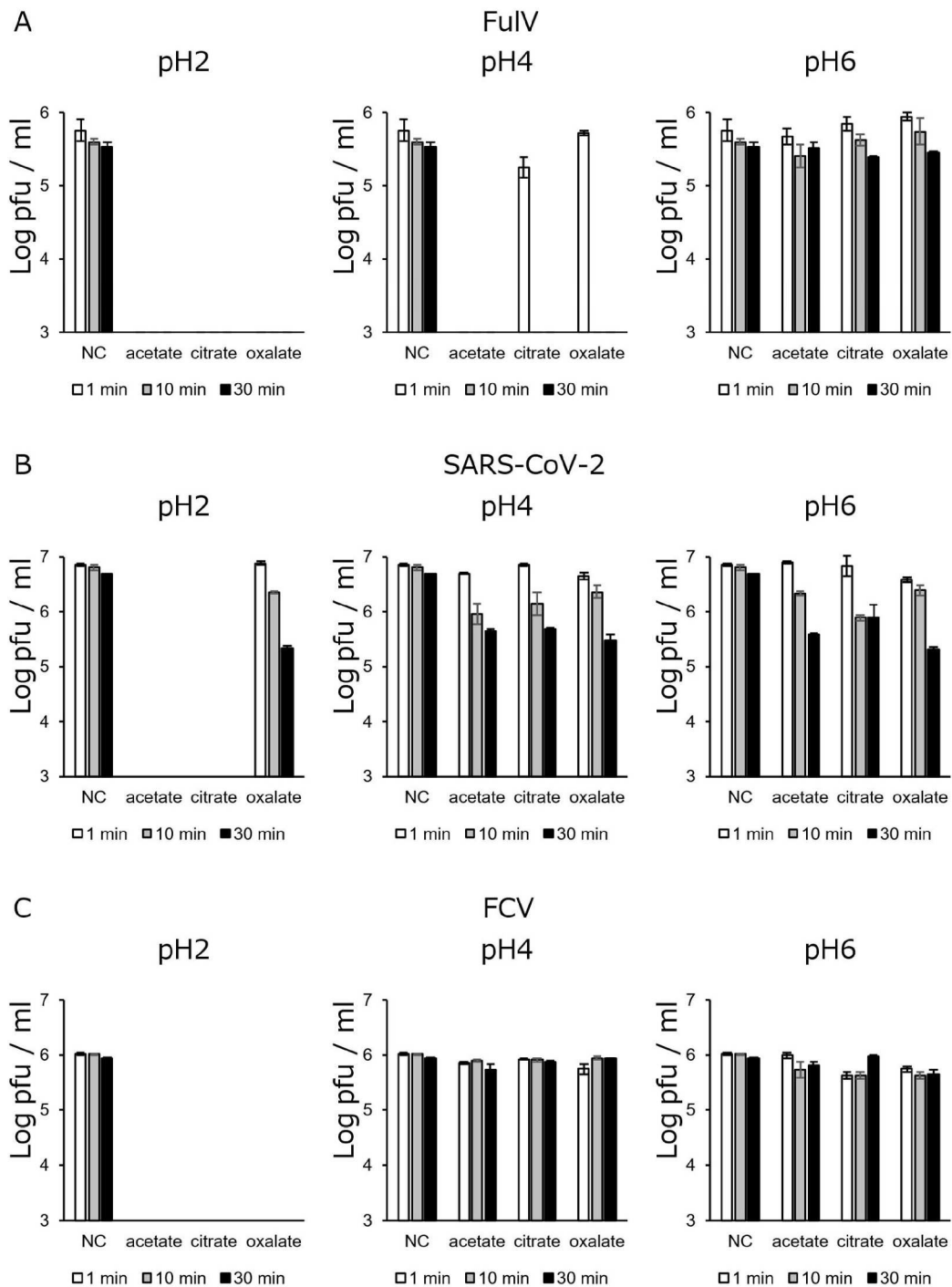


Fig. 2

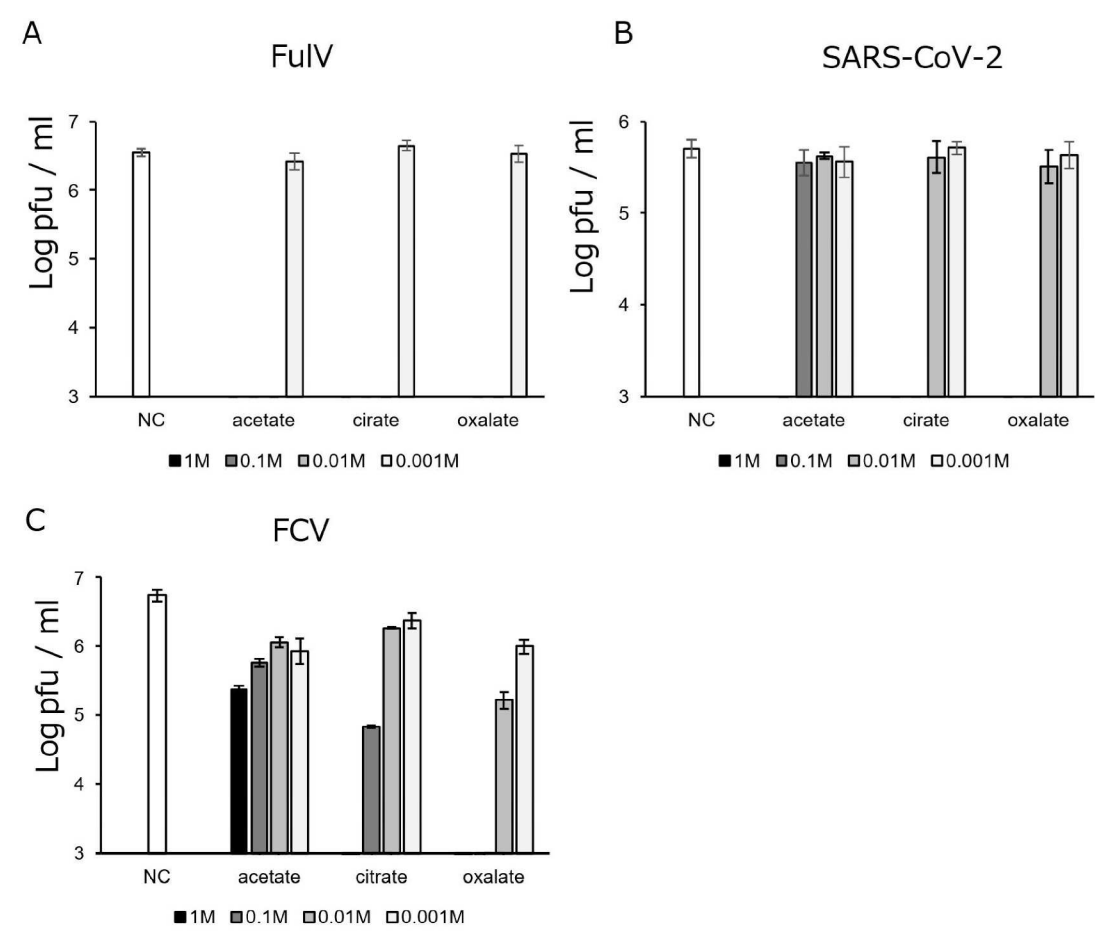
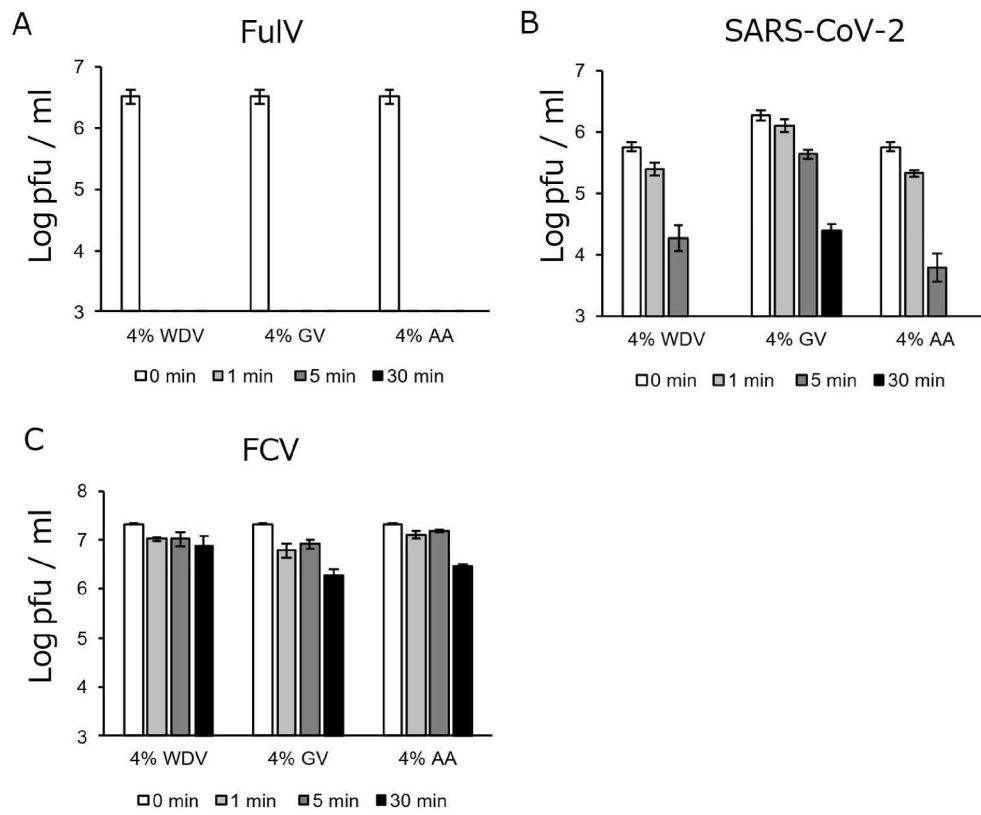


Fig. 3



2.9. Tables

Table 1 pH in the reaction mixtures of pH-dependent acids and 2%FBS DMEM

Acid	Original adjusted pH	pH in the reaction mixture*
Acetate	2.07	2.04 ± 0.12
	4	4.09 ± 0.01
	6.03	6.03 ± 0.09
Citrate	2.05	2.24 ± 0.02
	4.04	4.24 ± 0.10
	6	6.00 ± 0.15
Oxalate	2.02	2.02 ± 0.05
	3.96	4.09 ± 0.13
	6.01	6.77 ± 0.02

pH in original adjusted weak acids was measured, following to measure pH in mixture in triplicate.

*Average value in triplicate ± 95 % confidence interval.

The pH values in the reaction mixtures of molarity-dependent mixtures were 2.39–7.35 in acetic acid, 1.27–6.41 in citric acid, and 0.63–6.79 in oxalic acid (Table 2).

Table 2 pH in the reaction mixtures of molarity-dependent acids and 2%FBS DMEM

Acid	Molarity of acid	Original adjusted pH	pH in the reaction mixture*
Acetate	1 M	2.31	2.39 ± 0.090
	0.1 M	2.73	3.25 ± 0.100
	0.01 M	3.31	4.46 ± 0.258
	0.001 M	3.94	7.35 ± 0.259
Citrate	1 M	1.25	1.27 ± 0.094
	0.1 M	1.85	2.02 ± 0.112
	0.01 M	2.46	2.94 ± 0.169
	0.001 M	3.2	6.41 ± 0.080
Oxalate	1 M	0.59	0.63 ± 0.100
	0.1 M	1.17	1.20 ± 0.000
	0.01 M	1.97	2.25 ± 0.029
	0.001 M	2.95	6.79 ± 0.305

pH in original adjusted weak acids was measured, following to measure pH in mixture in triplicate.

* Average value in triplicate ± 95 % confidence interval.

2.10. Supplementary data

Table S1. pH values in diluent reaction solutions of pH-dependent acids and 2%FBS DMEM for plaque assays

Acid	pH	Diluents				
		1	0.1	0.01	0.001	0.0001
Acetic acid	pH2	2.21*	3.46*	4.99*	7.22	7.92
	pH4	4.18*	5.21*	7.27	7.95	7.95
	pH6	5.96*	7.87	8.25	8.26	8.23
Oxalic acid	pH2	1.92*	5.93*	7.93	8.32	8.27
	pH4	4.05*	6.95	8.1	8.21	8.2
	pH6	6.94	8.27	8.25	8.22	8.25
Citric acid	pH2	1.97*	3.12*	6.4	7.79	8.09
	pH4	4.13*	4.45*	6.06	7.51	8.09
	pH6	6.15	6.9	7.84	8.11	8.17

Reaction mixtures of weak acids (pH2, 4, 6) and 2%FBS DMEM (9:1) were followed to 10-fold serially dilution (1-0.0001) and pH of each diluent was measured. “*” indicated acidic pH in solutions.

Table S2. pH values in diluent reaction solutions of molarity concentration-dependent acids and 2%FBS DMEM for plaque assays

Acid	Molarity	Diluents				
	concentration	1	0.1	0.01	0.001	0.0001
Acetic acid	1M	2.6*	4.46*	7	8.15	8.03
	0.1M	3.28*	6.87	7.9	8.01	7.96
	0.01M	4.47*	8	8.13	8.16	8.11
	0.001M	7.19	8.16	8.18	8.13	8.13
Citric acid	1M	1.59*	2.91*	6.24	7.7	7.99
	0.1M	2.28*	6.27	7.8	8.07	8.04
	0.01M	3.34*	7.72	8.08	8.13	8.11
	0.001M	6.68	8.1	8.15	8.09	8.09
Oxalic acid	1M	1.03*	1.61*	6.16	7.58	7.87
	0.1M	1.49*	6.26	7.66	7.97	7.91
	0.01M	2.33*	7.63	7.97	7.93	7.8
	0.001M	6.63	8.04	8.03	8.03	7.98

Reaction mixtures of weak acids (1-0.001M) and 2%FBS DMEM (9:1) were followed to 10-fold serially dilution (1-0.0001) and pH of each diluent was measured. “*” indicated acidic pH in the solutions.

Table S3. pH values in diluent reaction solutions of edible vinegar and DMEM for plaque assays

Acid	Percentage	Original	Diluents				
		pH	1	0.1	0.01	0.001	0.0001
GV	4%	2.76*	2.67*	4.48*	7.39	8.17	8.61
WDV	4%	2.12*	2.55*	4.99*	8.1	8.55	8.82
AA	4%	2.23*	2.54*	4.76*	7.61	8.17	8.77

pH in original vinegar (4%GV, 4% WDV, 4% AA) was measured before mixing with 2%FBS DMEM.

Reaction mixtures of vinegar (4%GV, 4% WDV, 4% AA) and 2%FBS DMEM (9:1) were followed to 10-fold serially dilution (1-0.0001) and pH of each diluent was measured. “*” indicated acidic pH in the solutions.

Table S4. Quantification of cell viability in diluent reaction solutions of weak acids corresponding to pH value.

Acid		Diluents				
			0.1	0.01	0.001	0.0001
Acetic acid	pH		3.46	4.99	7.22	7.92
	Absorbance	Vero				
	[A _{450nm} -A _{655nm}]	E6/TMPRSS2	0.23*	0.20*	0.52	0.57
		CRFK	0.17*	0.34*	0.63	0.54
		MDCK	0.28*	0.59*	0.85	0.66
Oxalic acid	pH		5.93	7.93	8.32	8.27
	Absorbance	Vero				
	[A _{450nm} -A _{655nm}]	E6/TMPRSS2	0.25*	0.61	0.59	0.63
		CRFK	0.31*	0.70	0.72	0.82
		MDCK	0.34*	0.79	0.84	0.79
Citric acid	pH		3.12	6.4	7.79	8.09
	Absorbance	Vero				
	[A _{450nm} -A _{655nm}]	E6/TMPRSS2	0.26*	0.64	0.64	0.73
		CRFK	0.23*	0.58*	0.69	0.81
		MDCK	0.29*	0.78	0.87	0.87

pH of each diluent (0.1-0.0001) of weak acids (acetic acid, oxalic acid and citric acid at pH 2) were measured. Cell viability of the diluents were tested by XTT cell viability assay in triplicate. Negative control absorbance [A_{450nm}-A_{655nm}]: Vero E6/TMPRSS2=0.6, CRFK=0.67, MDCK=0.77. “*” indicated cell damage by weak acids diluents.

Table S5. Quantification of cell viability of diluent reaction solutions of vinegar corresponding to pH value.

Acid	pH	Dilutions				
			0.1	0.01	0.001	0.0001
4%	pH		4.48	7.39	8.17	8.61
GV	Absorbance	Vero	0.20*	0.55	0.56	0.67
	[A _{450nm} -	E6/TMPRSS2				
	A _{655nm}]					
		CRFK	0.27*	0.66	0.75	0.75
		MDCK	0.27*	0.83	0.80	0.73
4%	pH		4.99	8.1	8.55	8.82
WDV	Absorbance	Vero	0.19*	0.72	0.59	0.76
	[A _{450nm} -	E6/TMPRSS2				
	A _{655nm}]					
		CRFK	0.09*	0.64	0.68	0.76
		MDCK	0.25*	0.92	0.90	0.86
4%	pH		4.76	7.61	8.17	8.77
AA	Absorbance	Vero	0.17*	0.54	0.59	0.62
	[A _{450nm} -	E6/TMPRSS2				
	A _{655nm}]					
		CRFK	0.10*	0.50	0.57	0.59
		MDCK	0.27*	0.84	0.88	0.86

pH of diluents (0.1-0.0001) of vinegar (4%GV, 4%WDV, 4%AA) were measured. Cell viability of vinegar acids were tested XTT cell viability assay in triplicate. Negative control absorbance [A_{450nm}-A_{655nm}]: Vero E6/TMPRSS2=0.6, CRFK=0.67, MDCK=0.77. “*” indicated cell damage by diluents.

3. Chapter 2

**Virucidal effects of contact lens disinfectants
against enveloped and nonenveloped
pathogenic viruses causing conjunctivitis**

3.1. Abstract

Objectives: Contact lens (CL) contamination carries the risk of viral keratoconjunctivitis transmission, particularly to CL users. Inadequate disinfection and viral stability in CL disinfectants of Human adenovirus (HAdV-D) and Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can contribute to ocular infections. In this study, we evaluated the antiviral efficacy of commercial CL disinfectants against these viruses.

Methods: Prior to the experiment, the cytotoxicity of CL disinfectants was assessed in A549 and Vero E6/TMPRSS2 cells to determine the detection limits. Stock viruses and each CL disinfectant were mixed in a 1:9 ratio and incubated according to the manufacturer's protocol. After the reactions, infectious viral titers were determined and compared with those of mock samples without disinfectants.

Results: Sodium hypochlorite-and hydrogen peroxide-based CL disinfectants reduced both enveloped SARS-CoV-2 and nonenveloped HAdV-D56 to below the detection limits. Salicylic acid and some PHMB-based disinfectants effectively inactivated enveloped SARS-CoV-2 but not nonenveloped HAdV-D56. Other kinds of PHMB, ϵ -Polylysine, PQ-1 and alexidine hydrochloride, did not exhibit efficient inactivation against both viruses.

Conclusions: Sodium hypochlorite-and hydrogen peroxide-based CL disinfectants have potent virucidal activities against enveloped and nonenveloped viruses. HAdV is the most important causative agent of conjunctivitis. CL disinfectants are recommended to sanitize contact lenses on a daily basis, following the manufacturer's protocol.

3.2. Introduction

Contact lens (CL) contamination carries the risk of transmitting infectious diseases such as viral keratoconjunctivitis [141]. CL users with long-term and high frequency use including of overnight or extended-wear users face a increased risk of moderate to severe microbial keratitis [142,143]. According to a surveillance study conducted in the United States, 30.2% of wearers, including users of daily disposable, planned replacement soft lenses, overnight soft lenes, and rigid lenses experienced red or painful eye requiring medical care, often linked to risk behaviours [144]. Relevant risk factors for viral ocular infections include insufficient contact time with CL disinfectants and the ineffectiveness of CL disinfectants inactivating the virus. Viral conjunctivitis can spread through direct or indirect contact with contaminated CL surfaces and improper use of CL disinfectants, leading to ocular infections [145].

Human adenovirus (HAdV) is the main infectious agent of viral conjunctivitis [79]. Adenoviruses are double-stranded DNA viruses that are nonenveloped, known to cause a wide range of diseases, including ocular, respiratory, and gastroenteritis. HAdV species D (HAdV-D) is the primary causative agent of keratoconjunctivitis. This extremely resilient virus can persist on hands, fomites, and even metal and plastic surfaces for up to 49 days [76–78]. Since 2011, HAdV-D56 outbreaks have been reported in the USA and China, as well as conjunctivitis and urethritis in patients in Japan [72,73,146] .

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection may also lead to ocular manifestations, including conjunctivitis, which has been reported in clinics and by the World Health Organization (WHO) during the COVID-19 pandemic [147,148]. Coronaviruses are RNA viruses encapsulated in an enveloped structure. This envelope contains a lipid bilayer with spike glycoproteins that play a crucial role in receptor attachment.

Following the development of protection against microbial contamination of CL surfaces, a variety of lens-care systems have become commercially available. Alternative disinfection procedures for CL must be user-friendly and nonharmful to both the CL wearer and the lens material.

Commercial CL disinfectants include several types such as hydrogen peroxide, sodium hypochlorite and salicylic acid.

Enveloped viruses are generally considered to be more susceptible to environmental stress owing to their lipid membranes, whereas nonenveloped viruses exhibit greater resistance to harsh conditions due to physical properties of capsid protein. However, viral stability under environmental stress, such as acidity and other chemicals, varies depending on the viral characteristics or chemical compounds, regardless of the envelope structure. Our previous study reported that SARS-CoV-2 can resist low-pH oxalate environments [149]. However, comprehensive data on the viral stability are limited.

It has been shown that CL disinfectants are capable of inhibiting bacterial growth [150]. However, their virucidal properties have not been fully elucidated. In this study, the inactivation effects of CL disinfectants were examined against the enveloped virus SARS-CoV-2 and the nonenveloped virus HAdV-D, which causes conjunctivitis.

3.3. Materials and Methods

3.3.1. Viruses and cells

SARS-CoV-2 (hCoV-19/Japan/TY40-385/2022 strain), HAdV-D56 and VeroE6/TMPRSS2 cells were kindly provided by the National Institute of Infectious Diseases (NIID; Tokyo, Japan). SARS-CoV-2 and HAdV-D56 were propagated in VeroE6/TMPRSS2 and A549 cells, respectively. These cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JR Scientific, Woodland, CA, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Carlsbad, CA, USA) and maintained at 37 °C in 5% CO₂. Virus-infected cells were incubated at 37 °C in 5% CO₂ in DMEM supplemented with 2% FBS and penicillin streptomycin (FUJIFILM WAKO, Osaka, Japan). As for SARS-CoV-2, the supernatant was harvested from cell cultures then stored at -80 °C as stock source of viruses. HAdV-D56 were harvested after 3 freeze-thaw cycles, following to store at -80 °C as stock source of viruses. Experiments using infectious SARS-CoV-2 and HAdV-D56 were carried out in biosafety level (BSL)-3 and BSL-2 laboratories at Yamaguchi University following standard BSL guidelines.

3.3.2. Inactivation with CL disinfectant solution

The CL disinfectants listed in Table 1 were provided by Menicon Co., Ltd., because they offer a comprehensive range of CL disinfectants across all product categories. The stock virus and each CL disinfectant were mixed at a 1:9 ratio at room temperature. The procedure and incubation time for each CL followed the operational protocol for each product (Table 1). Reactions with hydrogen peroxide were performed in a specific container containing neutralizing platinum. Sterile saline was used as a negative control.

3.3.3. Plaque-forming assay (PFA)

The viral titer of SARS-CoV-2 was determined using PFA. Virus solutions were 10 times serially diluted with 2% FBS in DMEM and inoculated on confluent Vero E6/TMPRSS2 cells (100µl per well). After incubation at 37 °C in 5% CO₂ for 90 minutes, the inoculum was removed, and the cells were washed with DMEM. Next, 0.8% agarose (Seaplaque GTG Agarose; Lonza, Rockland, ME,

USA) was added to each well containing DMEM supplemented with 2% FBS and incubated at 37 °C in 5% CO₂. Plaque formations were observed 4 days post infection (dpi), followed by thorough cell staining with 1% crystal violet (FUJIFILM WAKO, Osaka, Japan). Viral titers were expressed as plaque-forming units (PFU) / ml.

3.3.4. Median tissue culture infectious dose (TCID₅₀) assay

The viral titer of HAdV-D56 was determined using TCID₅₀. Virus solutions were 10 times serially diluted with 2% FBS in DMEM and inoculated on confluent A549 cells (100µl per well). Virus-inoculated cells were incubated at 37 °C in 5% CO₂ for 72 h. The cytopathic effects (CPE) were observed and scored using an optical microscope. TCID₅₀ per ml (TCID₅₀ /ml) was calculated using the Behrens-Karber method.

3.3.5. Cell viability of CL disinfectants

CL disinfectants were 10 times serially diluted with 2% FBS in DMEM and inoculated into A549 and Vero E6/TMPRSS2 cells. Hydrogen peroxide disinfectants were neutralized using neutralizing platinum before application to the cell. Following incubating for 90 minutes at 37 °C in 5% CO₂ cells were fixed with 10% formalin and stained with 0.1% crystal violet. Cell staining was defined as no cell toxicity, whereas a well on the plain was defined as cell toxicity. The minimum dilution without cell toxicity was used to determine the detection limit (Table 2).

3.3.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.2.3. The activity of all the disinfectants was compared to the blank using an unpaired t-test, with statistical significance set at $P < 0.05$.

3.4. Results

We first evaluated the cytotoxicity of the diluted CL disinfectants against Vero E6/TMPRSS2 and A549 cells which were used to determine the detection limits of the viral titers of SARS-CoV-2 and HAdV-D56, respectively. Minimum dilutions (maximum concentration) of each CL disinfectant without cell damage were evaluated, and the detection limit of the virus titrations was determined (Table 2). Toxicities against Vero E6/TMPRSS2 and A549 cells varied between the original concentrations (10^0 dilutions) and 10^2 dilutions, indicating that the detection limits of virus titrations in each assay were 10^0 – 10^2 PFU/ml for SARS-CoV-2 and 10^1 – 10^3 TCID₅₀/ml for HAdV-D56 (Table 2).

Disinfectants with sodium hypochlorite, Progent ip1, Progent ip2, and Progent showed significant inactivation effects with below-detection limits against both SARS-CoV-2 and HAdV-D56 (Fig. 1A). Disinfectants with hydrogen peroxide, Smart Clean, Meni-Sept, Clear Care Plus Hydraglyde, and AO Sept Clear Care also showed significant virucidal effects below the detection limits against both HAdV-D56 and SARS-CoV-2 (Fig. 1B). These results indicate that sodium hypochlorite-and hydrogen peroxide-based CL disinfectants possess strong inactivation effects against both enveloped and nonenveloped viruses.

Four-season care, including salicylic acid, decreased the infectious titer of SARS-CoV-2 below the detection limits and that of HAdV-D56, although the effect did not reach a 99% reduction in the infectious titer (Fig. 2A). Among the disinfectants containing PHMB, O₂ care aminosora showed significant inactivation against SARS-CoV-2, whereas the effect was limited against HAdV-D56 (Fig. 2B). However, Bloom care, Epica cold, Solocare Aqua, Cleadew repair solution, and Menicare Plus did not exhibit a significant decrease in infectious virus titers, with > 99% reduction (Fig. 2B). These observations suggest that salicylic acid-based disinfectant and some kind of PHMB-based disinfectants has virucidal effects against enveloped SARS-CoV-2, but the effects were limited against nonenveloped adenovirus.

Menicare Pure (ϵ -Polylysine), Unique Ph (Polyquaternium-1), cleadew procare solution (PHMB and alexidine hydrochloride), and Acuvue Revitalens (Polyquaternium-1 and alexidine hydrochloride)

did not show significant inactivation effects, with $> 99\%$ reduction against SARS-CoV-2 and HAdV-D56 (Fig. 3). These observations suggest that these disinfectants are insufficient to inactivate both enveloped and nonenveloped viruses.

3.5. Discussion

In this study, we showed that sodium hypochlorite-and hydrogen peroxide-based CL disinfectants have potent virucidal activity against both enveloped and nonenveloped viruses that cause conjunctivitis. A previous study showed that 0.32% w/w sodium hypochlorite inactivates SARS-CoV-2 within 5 minutes [151] . In this study, CL disinfectants containing low concentrations of these compounds were also capable of inactivating enveloped SARS-CoV-2 and nonenveloped HAdV-D56 under the recommended treatment conditions. Of note, a previous study reported that hydrogen peroxide-based CL disinfectant showed limited efficacy against adenovirus [152] . On the other hand, our current findings demonstrated that hydrogen peroxide-based formulations of ACC, CCP, MS and SC exhibited significant virucidal effects. While the underlying reason for these different effects remains unclear from current data, they may be attributable to variations in adenovirus type, types of CL disinfectants or differences in experimental methodology. Sodium hypochlorite and hydrogen peroxide act as strong oxidizing agents by releasing free radicals into water [153,154], and this process is likely to damage the viral envelope, proteins, and nucleic acids. Although these CL disinfectants may be harmful to the skin and ocular mucosa, the neutralization process reduces the chemical concentration through rinsing, scrubbing in water, and degradation in a neutralizing vessel. Therefore, CL disinfectants, such as Progent and Clear Care Plus Hydraglyde, are highly effective at avoiding contamination by viral conjunctivitis agents.

Salicylic acid-based CL disinfectants efficiently inactivated enveloped coronaviruses but not nonenveloped adenoviruses. Salicylic acid is a beta-hydroxy acid that functions as a plant immunity signalling molecule to induce resistance against plant viruses, including the tobacco mosaic virus [104]. A recent report showed that handwash containing salicylic acid inactivates SARS-CoV-2 within 30 s [151]. Our study showed that salicylic acid (0.2%) in CL disinfectants could inactivate SARS-CoV-2, suggesting that CL disinfectants, such as Four seasons care is useful in preventing enveloped viral infections in CL wearers.

Although O₂ care aminosora showed significant inactivation effects against enveloped SARS-CoV-2, other PHMB disinfectants did not exhibit efficient inactivation against either enveloped or

nonenveloped viruses. PHMB is a cationic antimicrobial agent that is widely used in the healthcare, food, and water industries. Previous studies have also indicated that disinfectants containing PHMB at working concentration of 0.0205–0.14% exhibited variable inactivation rates of SARS-CoV-2, ranging from 30% to 100% after 5–10 minutes of exposure [155]. A previous study using the MS2 bacteriophage as a nonenveloped virus model suggested that nonenveloped viral particles aggregate upon exposure to PHMB, presumably because of alterations in phage surface hydrophobicity, thus hampering the virucidal activity of biguanides [156]. This may explain why nonenveloped viruses have a resistance strategy against PHMB exposure.

CL disinfectants containing ϵ -Polylysine, PQ-1 and alexidine hydrochloride solution (Menicare Pure, Unique Ph, Cleadew Procure solution, and Acuvue Revitalens) could not inactivate either SARS-CoV-2 or HAdV-D56. A previous study showed that PQ-1 and alexidine hydrochloride did not inactivate the mouse hepatitis virus [157]. Thus, the virucidal effects of CL disinfectants may be limited against conjunctivitis viruses such as adenovirus and coronavirus. However, previous studies showed that rub-and-rinse procedure effectively reduce the infectious titers of both adenovirus and coronavirus [152,158]. In addition, our preliminary experiments indicated that even a simple rinse with water effectively reduced infectious viral titers to below detection limit (data not shown). These findings suggest that rinse and rub-and-rinse procedures can provide significant virucidal effects. Although PHMB-, ϵ -Polylysine-, PQ-1-, and alexidine hydrochloride-based CL disinfectants exhibited limited viral inactivation effects, the regimen of OCA, BC, CRS, MP and CPS include the rinse procedures, and those of MP, UP and AR recommend the rub-and-rinse procedures. Therefore, when using these CL disinfectants, appropriate procedures following the regimens is critical to reduce the risk of contamination of infectious viruses.

HAdV is the most important agent in conjunctivitis, and SARS-CoV-2 has also been reported to cause ocular infections [182]. HAdV-D56 was effectively deactivated by low concentrations of sodium hypochlorite CL disinfectants and hydrogen peroxide-based disinfectants within the manufacturer-recommended treatment time. These results indicate that regular CL wearers must strictly follow the manufacturer's instructions when disinfecting CLs to prevent adenovirus ocular infection. It is noteworthy that our experimental data were obtained under controlled laboratory

conditions. In natural environments, the viral titer attached to surfaces may typically be lower than that of laboratory stock samples. Therefore, inactivation efficacy may vary between laboratory conditions and living environments. However, CL disinfectants are recommended to sanitize contact lens on a daily basis, following the manufacturing procedure and time. We believe that our results will not only offer useful information into the virucidal effects of CL disinfectants but also provide a foundation for future studies aimed at elucidating the mechanisms based on different types of disinfectants.

3.6. Figure legends

Fig. 1. (A) Inactivation effects of sodium hypochlorite-based Progent (Pro), Progent ip1(Pip1) and Progent ip2 (Pip2). (B) Inactivation effects of hydrogen peroxide-based AO sept Clear Care (ACC), Clear Care Plus Hydraglyde (CCP), Meni-Sept (MS) and Smart clean (SC) against SARS-CoV-2 and HAdV-D56. Mock is viral titer under same incubation times of each disinfectant listed in Table 1.

Each sample was tested triplicate. Error bar stands for standard error of mean.

Fig. 2. (A) Inactivation effect of salicylic acid-based Four seasons care (FSC). (B) Inactivation effect of PHMB-based O₂ care aminosora (OCA), Bloom care (BC), Menicare Plus (MP), Cleadew repair solution (CRS), Solocare Aqua (SA) and Epica acid (EC). Mock is viral titer under same incubation times of each disinfectant listed in Table 1. Each sample was tested triplicate. Error bar stands for standard error of mean.

Fig. 3. Inactivation effect of (A) Menicare Pure (MP) containing ϵ -Polylysine -based, (B) Unique Ph (UP) containing PQ-1 (polyquaternium-1), (C) Cleadew procare solution (CPS) containing alexidine hydrochloride and PHMB and (D) Acuvue Revitalens (AR) containing alexidine hydrochloride and PQ-1. Mock is viral titer under same incubation times of each disinfectant listed in Table 1. Each sample was tested triplicate. Error bar stands for standard error of mean.

3.7. Figures

FIG.1

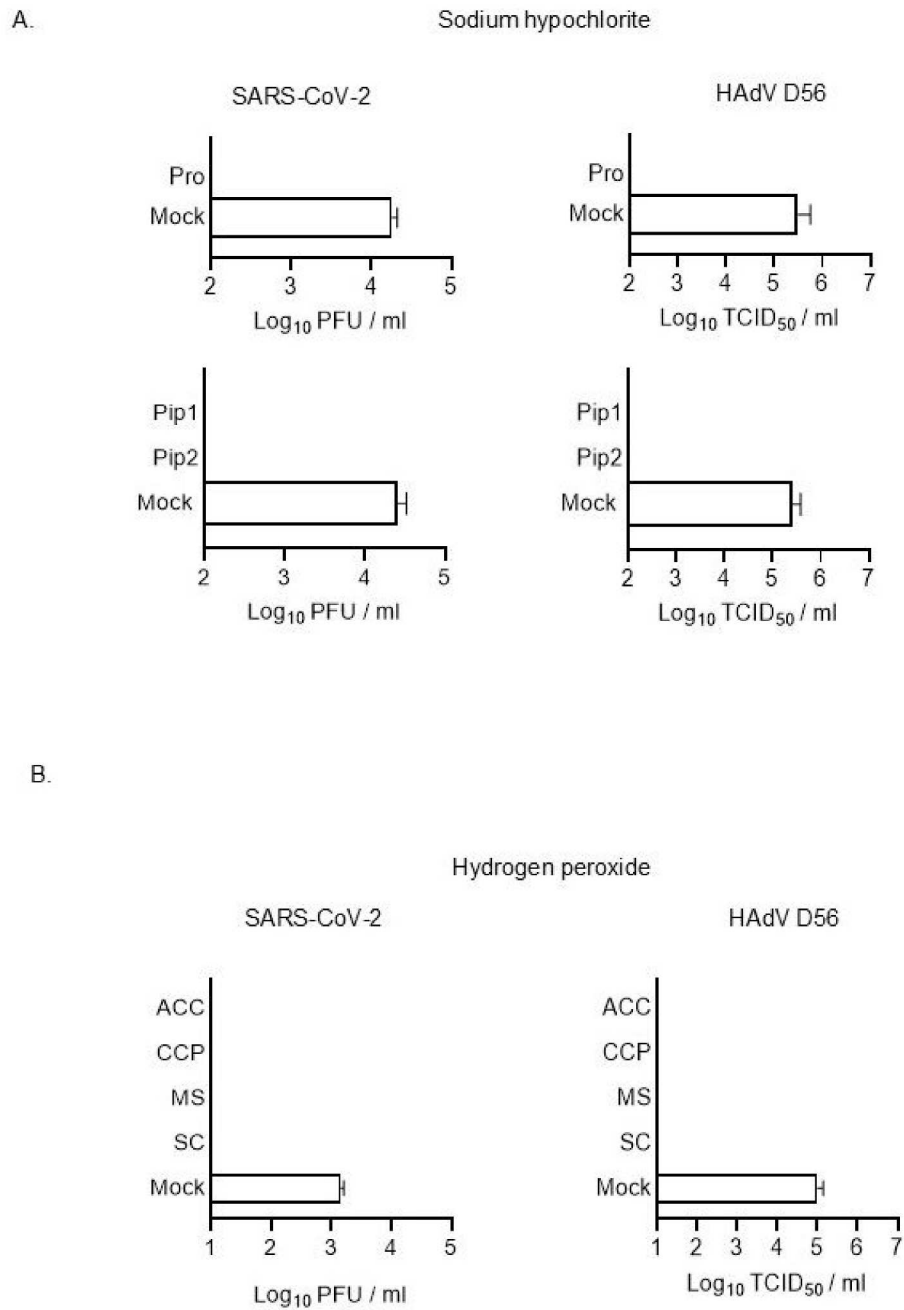
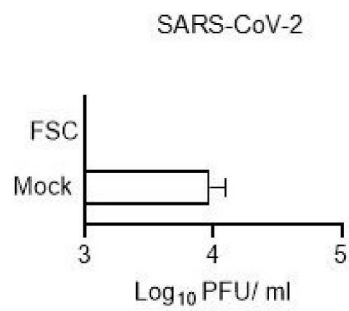
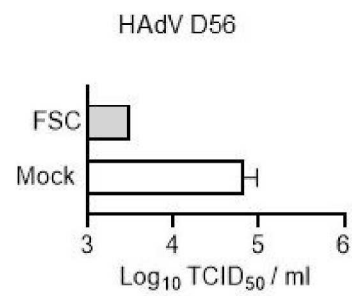


FIG.2

A.



Salicylic acid



B.

PHMB

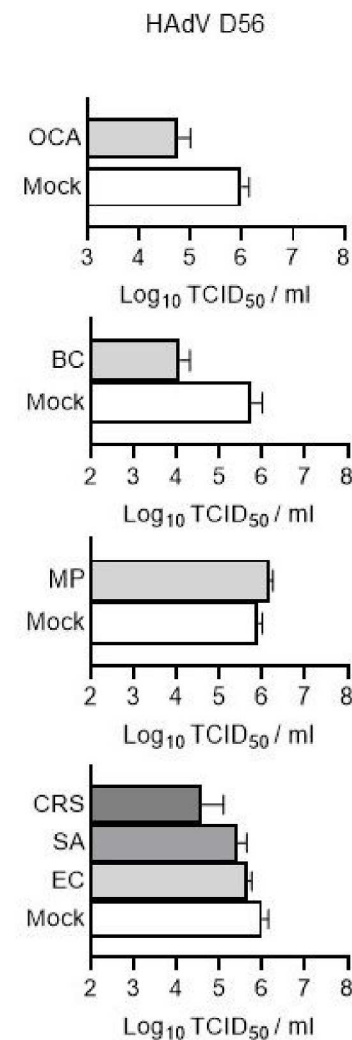
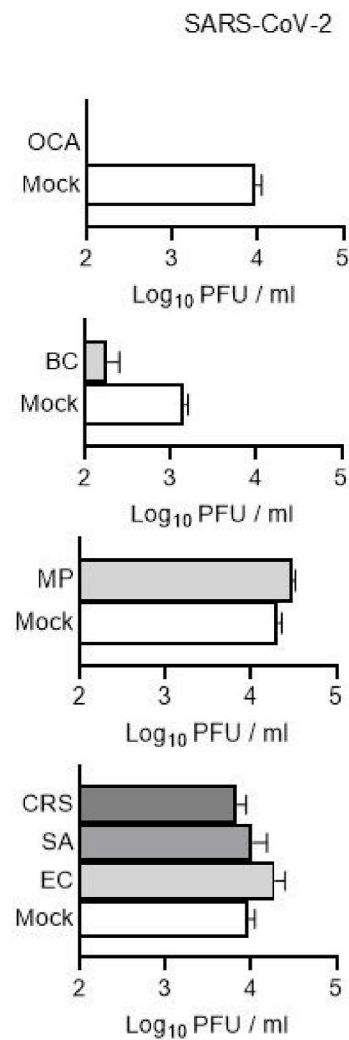
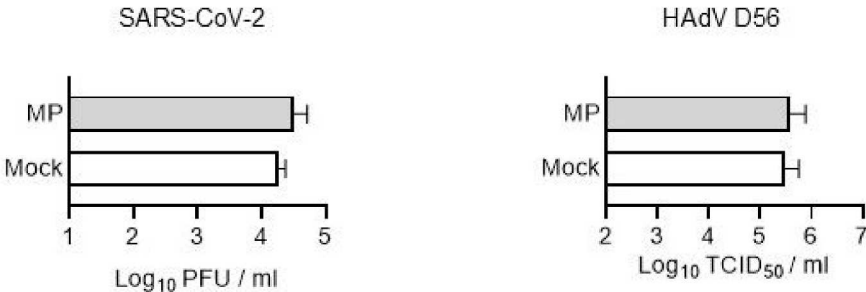


FIG.3

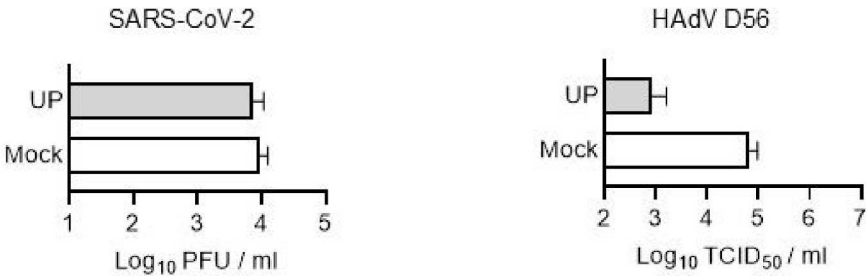
A.

ϵ -Polylysine



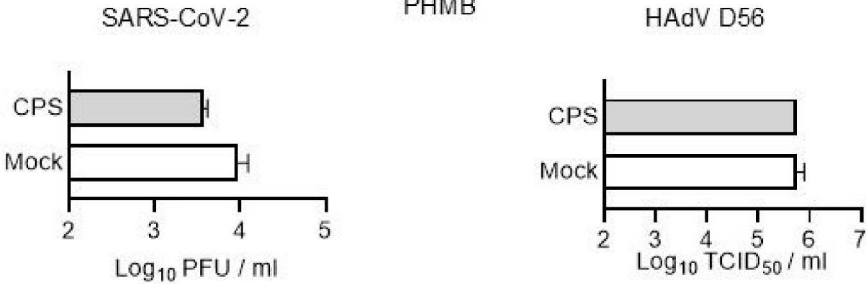
B.

PQ-1



C.

Alexidine hydrochloride,
PHMB



D.

Alexidine hydrochloride,
PQ-1



3.8. Tables

Table 1. CL disinfecting solutions used in this study.

Product name	Manufacturer	Component	Conc.	Time
Progent	Menicon		Ab.0.2%	30 mins
Progent ip1	Menicon	Sodium hypochlorite	Ab.0.1%	10 mins
Progent ip2	Menicon		Ab.0.067%	10 mins
AO sept Clear Care	Alcon		3.42%	6 h
Clear Care Plus	Alcon		3.42%	6 h
Hydraglyde		Hydrogen peroxide		
Meni-Sept	Menicon		3.42%	6 h
Smart clean	Menicon		3.42%	6 h
Four seasons care	Menicon	Salicylic acid	0.20%	4 h
O ₂ care aminosora	Menicon		100 ppm	4 h
Bloom care	Menicon nect		1 ppm	6 h
Menicare Plus	Menicon		5 ppm	5 mins
Cleadew repair solution	Ophtecs	PHMB*	1 ppm	4 h
Solocare Aqua	Menicon		1 ppm	4 h
Epica cold	Menicon		1 ppm	4 h
Menicare Pure	Menicon	ϵ -Polylysine	100 ppm	30 mins
Unique Ph	Menicon	PQ-1	10 ppm	4 h

cleadew procare solution	Ophtecs	Alexidine, PHMB	Alexidine 4ppm; PHMB 1ppm	4 h
Acuvue Revitalens	Johnson and Johnson	Alexidine hydrochloride, PQ- 1	Alexidine hydrochloride 0.0016m; PQ-1 0.003mg	4 h

*PHMB: Polyhexamethylene biguanide

*PQ-1: Polyquaternium-1

Ab: Acid base

Table 2. Cell toxicity of CL disinfecting solutions and viral detection limit

Products	Vero E6/TMPRSS2		A549 cell	
	Minimum dilution	Detection limit: (Log ₁₀ PFU/ ml)	Minimum dilution factor	Detection limit
	without cell toxicity (Log ₁₀)		to reduce cell toxicity (Log ₁₀)	Unit: Log ₁₀ TCID ₅₀ / ml
Progent	1	2	1	2
Progent ip1	1	2	1	2
Progent ip2	1	2	1	2
AO sept Clear	0	1	0	1
Care				
Clear Care Plus	0	1	0	1
Hydraglyde				
Meni-Sept	0	1	0	1
Smart clean	0	1	0	1

Four seasons care	2	3	2	3
O ₂ care				
aminosora	1	2	2	3
Bloom care	1	2	1	2
Menicare Plus	1	2	1	2
Cleadew repair solution	1	2	1	2
Solocare Aqua	1	2	1	2
Epica cold	1	2	1	2
Menicare Pure	0	1	1	2
Unique Ph	0	1	1	2
cleadew procare solution	1	2	1	2
Acuvue	1	2	1	2
Revitalens				

4. CHAPTER 3

Gastric acid resistances of animal gastrointestinal coronaviruses

4.1. Abstract

Gastrointestinal coronaviruses such as porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) cause gastrointestinal diseases in animals despite being enveloped viruses, implying that they can pass through the highly acidic gastric lumen. This study focuses on the low pH acid resistance of PEDV, TGEV and equine coronavirus (ECoV) in fasted-state simulated gastric fluid (FaSSGF). Both 1M citric and acetic acid at pH2 significantly inactivated PEDV, TGEV and ECoV, similar to SARS-CoV-2, whereas oxalic acid showed limited inactivation effects. Interestingly, PEDV, TGEV and ECoV retained infectivity in FaSSGF, while SARS-CoV-2 was rapidly inactivated. The inclusion of surfactants in FaSSGF did not affect the inactivation; however, pepsin in FaSSGF exhibited virucidal effects against PEDV, TGEV and ECoV. These results suggest that despite possessing an enveloped structure, PEDV, TGEV and ECoV exhibit notable resistance to low-pH gastric acid environments. Moreover, the higher susceptibility of PEDV and TGEV in piglets may be attributed to limited digestive enzyme activity, such as pepsin, because neonatal animals contain little to no proteolytic activity in the gastric lumen. Our findings suggest that gastrointestinal coronaviruses retain infectivity during gastrointestinal passage, and have potential for fecal–oral transmission by maintaining infectivity in low-pH gastric acid environments.

4.2. Introduction

Porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) cause acute gastrointestinal disease in piglets, leading to severe watery diarrhea with 50–100% mortality [9]. PEDV and TGEV infect piglets through rapid fecal-oral, fomite, and airborne transmission [18]. PEDV NK94P6Tr- was isolated in the 1990s in Japan and has been used as antigen for swine surveillance in Japan [159]. TGEV TO-163 strain was isolated from the first outbreak in Toyama, Japan in 1956 using CPK cells [160].

PEDV and TGEV belong to the family *Coronaviridae*, genus *Alphacoronavirus*, species *Alphacoronavirus porci* and *Alphacoronavirus suisi*, respectively [9]. Coronaviruses (the family *Coronaviridae*) are enveloped, positive-sense, single-strand RNA viruses [10], and are taxonomically classified into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [161]. Alphacoronavirus and Betacoronavirus mainly infect mammals, whereas Deltacoronavirus and Gammacoronavirus primarily infect avian species with some exceptions in mammals [162].

Most viruses responsible for gastrointestinal are non-enveloped, as their proteinaceous capsid structures confer resistance to acidic environment. In the mammalian stomach, gastric fluid consists primarily of hydrochloric acid (HCl) and sodium chloride (NaCl), which together maintain a highly acidic environment essential for food digestion and the inactivation of ingested pathogens. The gastric environment in the fasted stage typically exhibits a pH of 1 to 2 [110,163], acting as a primary barrier against microbial invasion and potentially destabilizing the lipid-enveloped viruses [164].

Interestingly, despite their lipid enveloped structure, coronaviruses present a notable exception to this paradigm [165], and several coronaviruses including PEDV and TGEV cause gastrointestinal infections in livestock animals. Equine coronavirus (ECoV), belonging to the genus *Betacoronavirus*, also causes gastrointestinal signs in horses [39]. ECoV caused outbreaks at Obihiro Racecourse in Hokkaido, Japan, in 2004, 2009 and 2012, and the virus was isolated from diarrheal samples using HRT-18G cells [37,38]. However, data on the acid resistance of coronaviruses remain limited compared to non-enveloped viruses. Therefore, understanding viral stability in acidic environments is

essential for assessing the potential for oral transmission and for developing future antiviral drug strategies and infection control.

In a previous study, we showed that the effectiveness of weak acids in inactivating non-enveloped and enveloped viruses including Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) varies even at the same pH [149]. Acetic and citric acids inactivated SARS-CoV-2 at pH 2, whereas oxalic acid did not, suggesting that the virucidal effect of weak acids is not determined solely by pH, but that additional factors may also influence these effects. In this study, we investigated the acid resistance of gastrointestinal coronaviruses of PEDV, TGEV and ECoV, focusing on their inactivation kinetics in gastric acidic environments.

4.3. Materials and Methods

4.3.1. Cells and virus

HRT-18G cells were obtained from American Type Culture Collection (ATCC). VeroE6/TMPRSS2 cells were kindly provided by the National Institute of Infectious Diseases (NIID; Tokyo, Japan). Human rectal tumor 18G (HRT-18G), the porcine kidney cell (CPK), African green monkey kidney cell (Vero E6) and VeroE6 cells expressing the transmembrane serine protease TMPRSS2 (VeroE6/TMPRSS2), Madin-Darby canine kidney (MDCK), Vero 9013 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, Canada) supplemented with penicillin-streptomycin (FUJIFILM WAKO, Osaka, Japan), and 10% fetal bovine serum (FBS; JR Scientific, Woodland, CA, USA). The cells were maintained at 37 °C with 5% CO₂. PEDV NK94P6Tr- and TGEV TO-163 strain were kindly provided from Central Livestock Hygiene Service Center in Yamaguchi Prefecture. ECoV isolated from the feces of an ECoV-infected horse was kindly provided by the Tokachi Livestock Hygiene Service Center in Hokkaido, Japan. SARS-CoV-2 (hCoV-19/Japan/TY40-385/2022 strain) was kindly provided by NIID. Coxsackievirus B2 (CVB2) and Influenza A/H1N1 (FulV) were kindly provided by the Yamaguchi Prefecture Institute of Public Health and Environment (Yamaguchi, Japan). PEDV, TGEV, ECoV, FulV, CVB2 and SARS-CoV-2 were propagated in Vero E6, CPK, HRT-18G, MDCK, Vero 9013, and VeroE6/TMPRSS2 cells, respectively, using DMEM containing 2% FBS. Virus supernatants were harvested from cell cultures then stored at -80 °C as stock source of viruses. The SARS-CoV-2 was used at biosafety level (BSL)-3, and the others were handled in BSL-2 laboratories at Yamaguchi University following standard BSL guidelines.

4.3.2. Plaque-forming assay (PFA)

The infectious viral titers of PEDV, TGEV, SARS-CoV-2, FulV, and CVB2 were determined using a PFA. Briefly, each type of cell was grown in 24-well plates until confluent; subsequently, serially diluted viruses were inoculated (125 µL per well) in 90 mins. PEDV, TGEV, SARS-CoV-2 and CVB2 were diluted in DMEM supplemented with 2% FBS, and FulV was diluted in DMEM supplemented with 0.001% trypsin (trypsin from Hog pancreas 1:250; NACALAI TESQUE, Tokyo, Japan) and

0.2% bovine serum albumin (BSA; FUJIFILM WAKO, Osaka, Japan). After incubation, the inoculum was removed, and the cells were washed with DMEM. Subsequently, 0.8% agarose (SeaPlaque GTG Agarose; Lonza, Basel, Switzerland) in DMEM with 10% FBS or DMEM with 0.001% trypsin and 0.2 % BSA was added to each well, and incubated at 37 °C until visible plaques appeared. Plaque formations were observed at 2 days post-infection (dpi) for SARS-CoV-2, FulV, and CVB2, 3 dpi for TGEV, and 5 dpi for PEDV. The addition of trypsin and BSA to the media did not influence pH of reaction samples for FulV, including acids and plaque formation. To retrieve the plate, the cells were fixed with 10% formalin and thoroughly stained with 1% crystal violet (FUJIFILM WAKO, Osaka, Japan). The viral titers were expressed as pfu/mL.

4.3.3. Median tissue culture infectious dose (TCID₅₀) assay

The viral titer of ECoV was determined using the TCID₅₀ method. Virus solutions were serially diluted 10-fold with 2% FBS DMEM and inoculated on confluent HRT-18G cells (100µl per well). Virus-inoculated cells were incubated at 37 °C in 5% CO₂ for 72 hr. The cytopathic effects were observed and scored using an optical microscope. The viral titers were expressed as TCID₅₀ /mL calculated using the Behrens–Kärber method.

4.3.4. Acids preparation and neutralization

1M acetic acid, citric acid, and oxalic acid at pH 2 were prepared by diluting stock solutions in sterile Milli-Q water and adjusting the pH with sodium hydroxide. Fasted-state simulated gastric fluid (FaSSGF) (Biorelevant, London, United Kingdom) (1M, pH 1.6) containing HCl and NaCl was prepared at room temperature following the manufacturer's protocol. FaSSGF powder containing surfactants (sodium taurocholate and lecithin) was stored at 4 °C. FaSSGF containing surfactants (FaSSGF surfactant) was prepared by adding 0.06 g/L of 3F powder (Biorelevant, London, United Kingdom) into FaSSGF. FaSSGF with pepsin (FaSSGF pepsin) was prepared by dissolving pepsin (Pepsin from porcine gastric mucosa; Sigma, Tokyo, Japan) at 0.1, 0.3, 0.4 and 0.8 mg/mL, and 0.06 g/L surfactants into FaSSGF. The pH was confirmed immediately after preparation using LAQUAtwin compact pH meter (HORIBA, Kyoto, Japan). The addition of surfactant or pepsin in FaSSGF in FaSSGF have no effect on sample pH and plaque formation. The reaction solution of

acetic acid was neutralized with 234 μ L, oxalic acid with 34 μ L, citric acid with 200 μ L of 10M sodium hydroxide (NaOH), FaSSGF was neutralized with 25 μ L of 1M NaOH. Neutralizing volume of NaOH was calculated prior to experiment.

4.3.5. Assessment of cytotoxicity of weak acids and FaSSGF

Weak acids, including 1M acetic acid, oxalic acid, and citric acid at pH 2, FaSSGF at pH 1.6 were mixed with 2%FCS DMEM in the ratio of 9:1, followed by neutralization with sodium hydroxide and then applied to cultured cells including CPK, Vero E6/TMPRSS2, Vero E6, HRT-18G, MDCK and Vero 9013 cell lines at both original and 10-fold diluted concentrations. For HRT-18G cell, reaction solution was incubated on cell for 24 hr at 37 °C in 5% CO₂. For other cell lines, incubation was 90 min. After a 24 hr or 90 min incubation, supernatant was removed, and the cells were fixed with 10% formalin and stained with 1% crystal violet (FUJIFILM WAKO, Osaka, Japan). The addition of surfactants in FaSSGF did not influence cytotoxic effect. The highest dilution without cytotoxic effect was determined as the detection limit of virus quantification. The 90-min incubated groups were to determine for PFA, 24-hour incubation groups were used for the TCID₅₀ assay.

4.3.6. Assessment of virucidal reactions of weak acids and FaSSGF

Viruses were mixed with oxalic acid, citric acid, acetic acid, FaSSGF and FaSSGF surfactant at the ratio of 1:9 and incubated at room temperature for 1, 30, 60, 90 mins. After reactions, sodium hydroxide was added to neutralize the pH to terminate the virucidal reaction, and were placed on ice for 5 mins. Sterilized Mili-Q water was used in mock treatment. PEDV was cultured for 5 dpi, TGEV and ECoV for 3 dpi; CVB2, FulV, and SARS-CoV-2 for 2 dpi, all maintained at 37 °C with 5% CO₂. Infectious viral titers were determined by PFA and TCID₅₀.

4.3.7. Statistical analyses

Statistical analyses were performed using GraphPad Prism 10.2.3. Disinfectants activity was compared to the control using an unpaired t-test, with statistical significance set at $P < 0.05$.

4.4. Results

Prior to inactivation experiments, we first assessed the cytotoxic effects of each acid to determine the detection limits of each virus titration using each cultured cell line. Neutralized acid samples were serially diluted and applied to each cell line, after which cell death was observed. Samples of acetic, citric, and oxalic acid were required 100-fold dilutions to exhibit non-toxicity for VeroE6/TMPRSS2, Vero E6, CPK and HRT-18G cells (Table 1). Thus, detection limits were 10^3 pfu/ml (SARS-CoV-2, PEDV, TGEV) or 10^3 TCID₅₀/mL (ECoV). On the other hand, FaSSGF did not show cytotoxicity in most cells without dilutions of neutralized acid samples, except HRT-18G cell lines (Table 1), indicating that detection limits of 10 pfu/mL (SARS-CoV-2, PEDV, TGEV, FulV and CVB2) or 10^2 TCID₅₀/mL (ECoV).

Our previous study demonstrated that SARS-CoV-2 was significantly inactivated by acetic and citric acids with more than $10^{3.83}$ pfu reductions compared with untreated controls after 1 min at pH 2, whereas inactivation effect of oxalic acid was comparatively limited with almost no reductions at 1 min and $10^{1.34}$ pfu reduction at 30 min [149].

Here, we examined the virucidal effects of weak acids of acetic acid, citric acid and oxalic acid against PEDV, TGEV and ECoV under pH 2 condition. Acetic acid and citric acid significantly reduced the virus titers of PEDV, TGEV and ECoV to below detection limits within 1 min as same as SARS-CoV-2 (Fig. 1). Interestingly, virus titers of PEDV, TGEV and ECoV were not significantly reduced by oxalic acid after 1 min (Fig. 1). Although virus titers of PEDV, TGEV and ECoV were under detection limits after 30 min, those reductions were $>10^{1.19}$ pfu/mL, $>10^{1.19}$ pfu/mL, and $>10^{1.67}$ TCID₅₀ /mL similar to that of SARS-CoV-2 with $>10^{1.34}$ pfu/mL (Fig. 1). These results indicated that TGEV, PEDV and ECoV were immediately inactivated by low pH 2 acids of acetic acid and citric acid, although those effects of oxalic acid were limited, suggesting that TGEV, PEDV and ECoV are susceptible to some kinds of acids at low pH conditions.

We next examined viral inactivation under simulated gastric acids conditions using FaSSGF containing HCl and NaCl at pH 1.6. Remarkably, the virus titers of TGEV, PEDV and ECoV were not significantly reduced after 1 min compared with their controls, and the virus titers were somewhat

reduced after 30 mins, but the reductions were very limited, with less than $10^{1.12}$ pfu/mL, $10^{1.69}$ pfu/mL, and $10^{1.74}$ TCID₅₀ /mL even after 90 min (Fig. 2). On the other hand, interestingly, SARS-CoV-2 was significantly inactivated to below detection limits for 1 min (Fig. 2), indicating that SARS-CoV-2 appears to be more vulnerable in FaSSGF. Enveloped FulV was also immediately inactivated in 1 min (Fig. 2). Of note, the non-enveloped CVB2 did not show significant reductions of infectious titers even after 90 min (Fig. 2), suggesting that non-enveloped viruses show significant acid resistance in FaSSGF.

These observations suggest that TGEV, PEDV, and ECoV possess infectious stability in gastric acid conditions at extremely low pH of 1.6, whereas infectivity of SARS-CoV-2 was readily diminished in this condition. Therefore, despite the same coronavirus group, it is suggested that gastrointestinal coronavirus such as TGEV and PEDV retain infectivity during gastrointestinal passage, potentially contributing to fecal-oral or enteric transmission routes.

The gastric lumen also contains surfactants that play a crucial role in the breakdown and emulsification of ingested materials, and these surfactants have been reported to affect virus infectivity [166,167]. Therefore, we also examined the influence of the surfactants in the FaSSGF surfactant. PEDV, TGEV, and ECoV maintained detectable infectivity in the FaSSGF surfactant even after 90 min of treatment (Fig. 3), similar to the results of FaSSGF alone. On the other hand, SARS-CoV-2 was significantly inactivated to below the detection limit in the FaSSGF surfactant within 1 min (Fig. 3). The enveloped FulV was also immediately inactivated in 1 min, whereas the non-enveloped CVB2 showed significant acid resistance over 90 mins in the FaSSGF surfactant (Fig. 3). These results suggest that surfactants did not affect the infectious stability of gastrointestinal coronaviruses such as PEDV and TGEV, and these coronaviruses could survive through gastric acids regardless of the presence of surfactant.

Pepsin is a representative digestive enzyme in the animal stomach. It is a proteolytic peptidase that activates in acidic environments, raising the possibility of virucidal effects in the gastric lumen. Thus, we next examined the inactivation effects of various concentration of pepsin in FaSSGF against coronaviruses. Interestingly, after incubation with FaSSGF pepsin of more than 0.1 mg/mL, PEDV was immediately inactivated to below detection limits in 1 min (Fig. 4). Virus titers of TGEV

significantly decreased depending on pepsin concentration within 1 min and were inactivated to below detection limits within 30 min (Fig. 4). ECoV titers also decreased depending on pepsin concentration in 1 min and inactivated to below detection limits in 30 min (Fig. 4). SARS-CoV-2 and FulV were inactivated below detection limits within 1 min (Fig. 4). On the other hand, inactivation effects against CVB2 were not observed in 30 min even at 0.8 mg/mL pepsin (Fig. 4). These observations suggest that the digestive enzyme pepsin exhibit virucidal effects against enveloped viruses including PEDV and TGEV in the acidic condition of the gastric lumen.

4.5. Discussion

In this study, we showed that despite possessing an enveloped structure, gastrointestinal coronaviruses of PEDV, TGEV and ECoV exhibit a notable ability to withstand low pH conditions in gastric acid environments, whereas SARS-CoV-2 was immediately inactivated. In general, oral infectious viruses are non-enveloped viruses such as norovirus and rotavirus, hepatitis A and hepatitis E viruses [168,169], and enveloped viruses seem to be vulnerable to disinfectants including low acid solutions due to the lipid layer construction. Therefore, our observations raise the possibility of a specific structure in the envelope of gastrointestinal coronavirus that confer resistance to low gastric pH enabling oral infections. Our findings will provide an important clue to elucidate the mechanism of oral infections due to enveloped viruses.

It is noteworthy that FaSSGF pepsin exhibit virucidal effects against PEDV, TGEV and ECoV, whereas FaSSGF surfactants did not affect the resistance to low gastric pH. Gastric pH and pepsin activity are influenced by age and food buffering effect, and adult animals have more developed gastric lumen with robust pepsin production and activity[170]. In contrast, neonatal animals contain little or no proteolytic activity in gastric lumen, with both acid and pepsin secretory capacities increases with age and weaning [171,172]. Therefore, the greater infectivity with PEDV and TGEV in piglets may be explained by the insufficient activity of digestive enzymes such as pepsin.

Interestingly, our previous observations suggested that the inactivation effects at pH 2 against SARS-CoV-2 were different between weak acids including acetic, citric and oxalic acids [149], and the current study also showed similar inactivation effects of weak acids against PEDV, TGEV and ECoV. It is considered that the virucidal effect against coronavirus is likely driven by conformational changes in viral proteins, particularly the spike protein, and virus exposure to low pH levels could eliminate viral infectivity. On the other hand, low pH is known to facilitate viral entry by inducing structural rearrangements for virus-membrane fusion, thereby leading to viral infection on host cell [173,174]. This discrepancy of infectivity at low pH raises the possibility that conformational change patterns are affected not only by pH, but also by other factors, such as the chemical properties of the acid and the endosomal environment.

Although coronaviruses show similar acids resistance to low pH in weak acids, PEDV, TGEV and ECoV retained their infectivity following exposure to gastric acid conditions. Specific structural features of the lipid envelope and the trimeric spike glycoprotein may relate to the different susceptibility against low acid conditions. Previous studies on coronaviruses have suggested that the conformational flexibility of the spike glycoprotein may contribute to viral survivability under acidic conditions [175–177]. A cryo-electron tomography study revealed that exposure of SARS-CoV-2 to highly acidic environments ($< \text{pH } 3$) induce partial unfolding of the spike protein, which impairs its ability to bind host cell receptors and initiate infection [178]. Therefore, PEDV, TGEV and ECoV may possess spike proteins with enhanced structural stability that preserve receptor-binding functionality even under extreme pH stress, distinguishing them from SARS-CoV-2 in their ability to maintain infectivity in the gastrointestinal environment.

In addition, the integrity of the viral lipid bilayer may be attributed to its unique lipid composition, which is often enriched in ordered, raft-associated lipids such as sphingolipids and cholesterol, forming a more rigid and stable membrane structure [179–181]. Such compositional differences could explain why PEDV, TGEV and ECoV maintain integrity and infectivity under low-pH conditions where SARS-CoV-2 may not. Further structural and biochemical investigations under low pH will be required to characterize pH-induced conformational changes in the coronaviral spike and envelope. These further studies will provide the important insights of not only fundamental aspects of various coronavirus characteristic in acid but also inform antiviral design strategies targeting structural stability in gastrointestinal environments.

5.5. Figure legends

Figure 1. Inactivation effects of 1M acetic acid (AA), citric acid (CA) and oxalic acid (OA) against PEDV, TGEV, ECoV and SARS-CoV-2 at pH 2 for 1 and 30 min. Negative control (NC) was incubated with sterile distilled water instead of weak acids. Error bars represent the standard error of triplicate measurements. Asterisk represented $P<0.05$.

Figure 2. The acid resistance of TGEV, PEDV, ECoV, SARS-CoV-2, enveloped FulV and non-enveloped CVB2 in FaSSGF (Fa) at pH 1.6 in 1, 30, 60 and 90min. Negative control (NC) was incubated with sterile distilled water instead of weak acids. Error bars represent the standard error of triplicate measurements. Asterisk represented $P<0.05$.

Figure 3. The acid resistance of coronaviruses in FaSSGF surfactant (FS) at pH 1.6 for 1, 30, 60 and 90 min. Negative control (NC) was incubated with sterile distilled water instead of weak acids. Error bars represent the standard error of triplicate measurements. Asterisk represented $P<0.05$.

Figure 4. The acid resistance of coronaviruses in 0.1, 0.3, 0.4, 0.8 mg/ml of FaSSGF pepsin (FP) at pH 1.6 for 1, 30, 60 and 90 min. Negative control (NC) was incubated with sterile distilled water instead of weak acids. Error bars represent the standard error of triplicate measurements. Asterisk represented $P<0.05$.

5.6. Figures

Fig.1

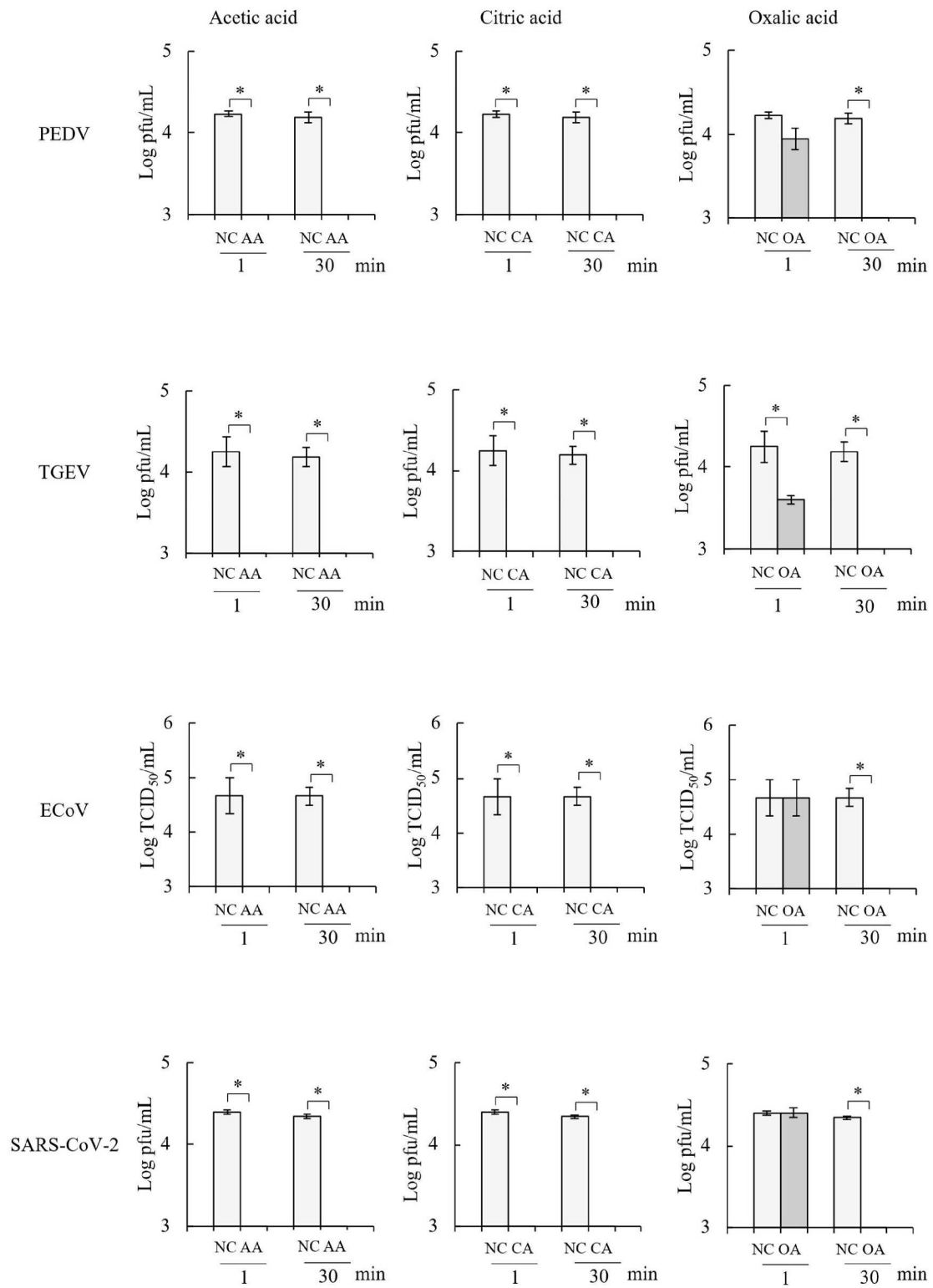


Fig.2

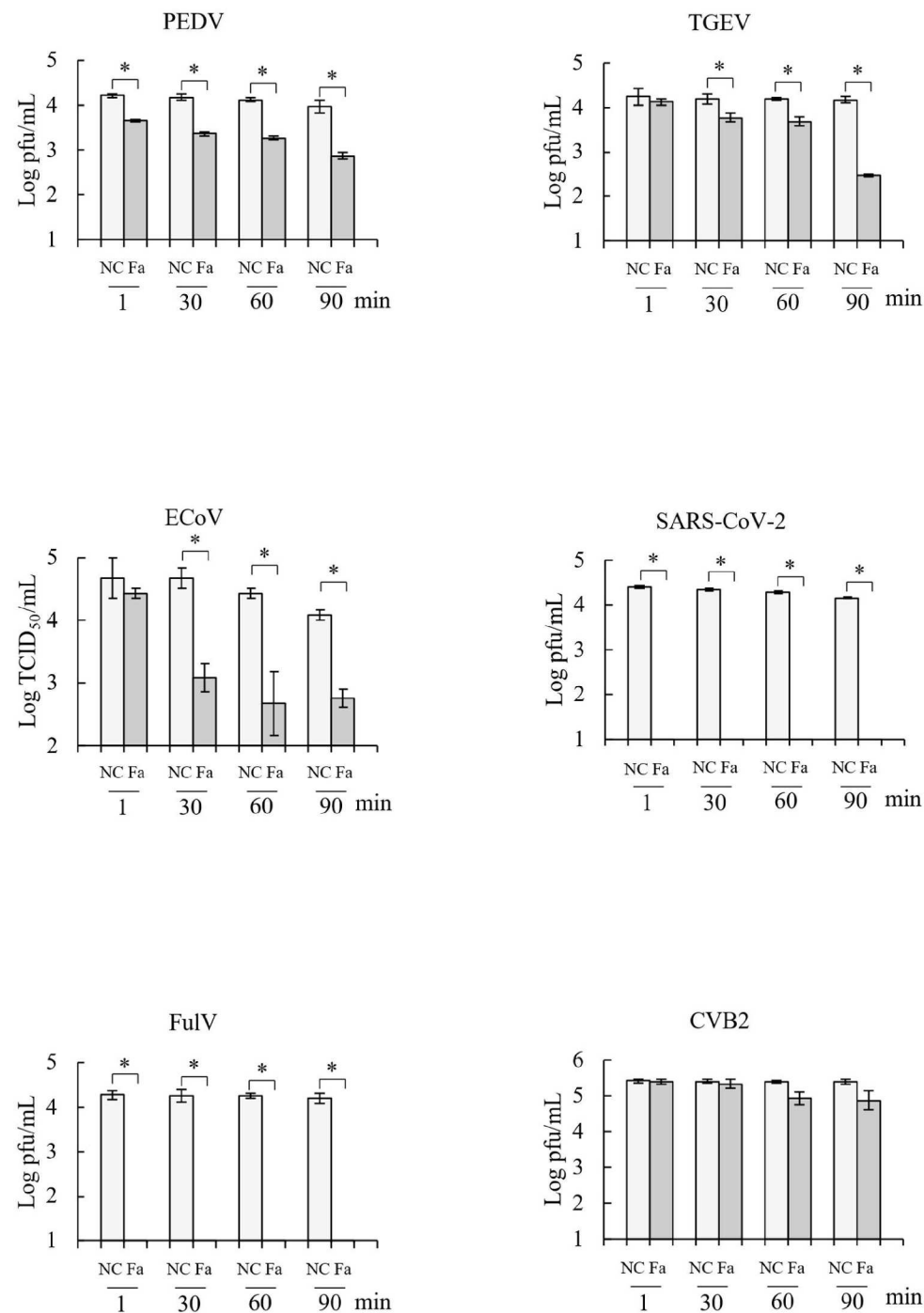


Fig.3

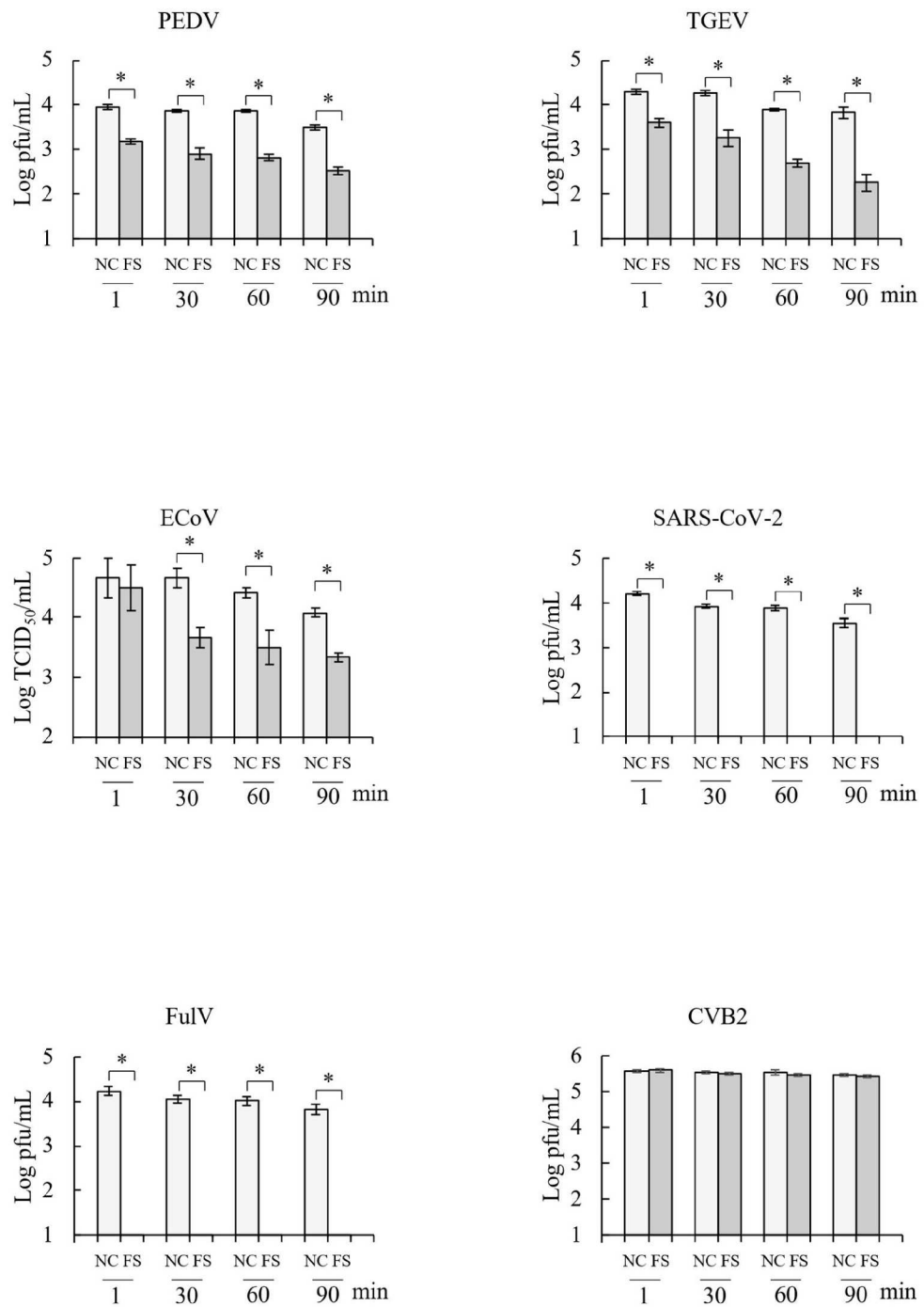
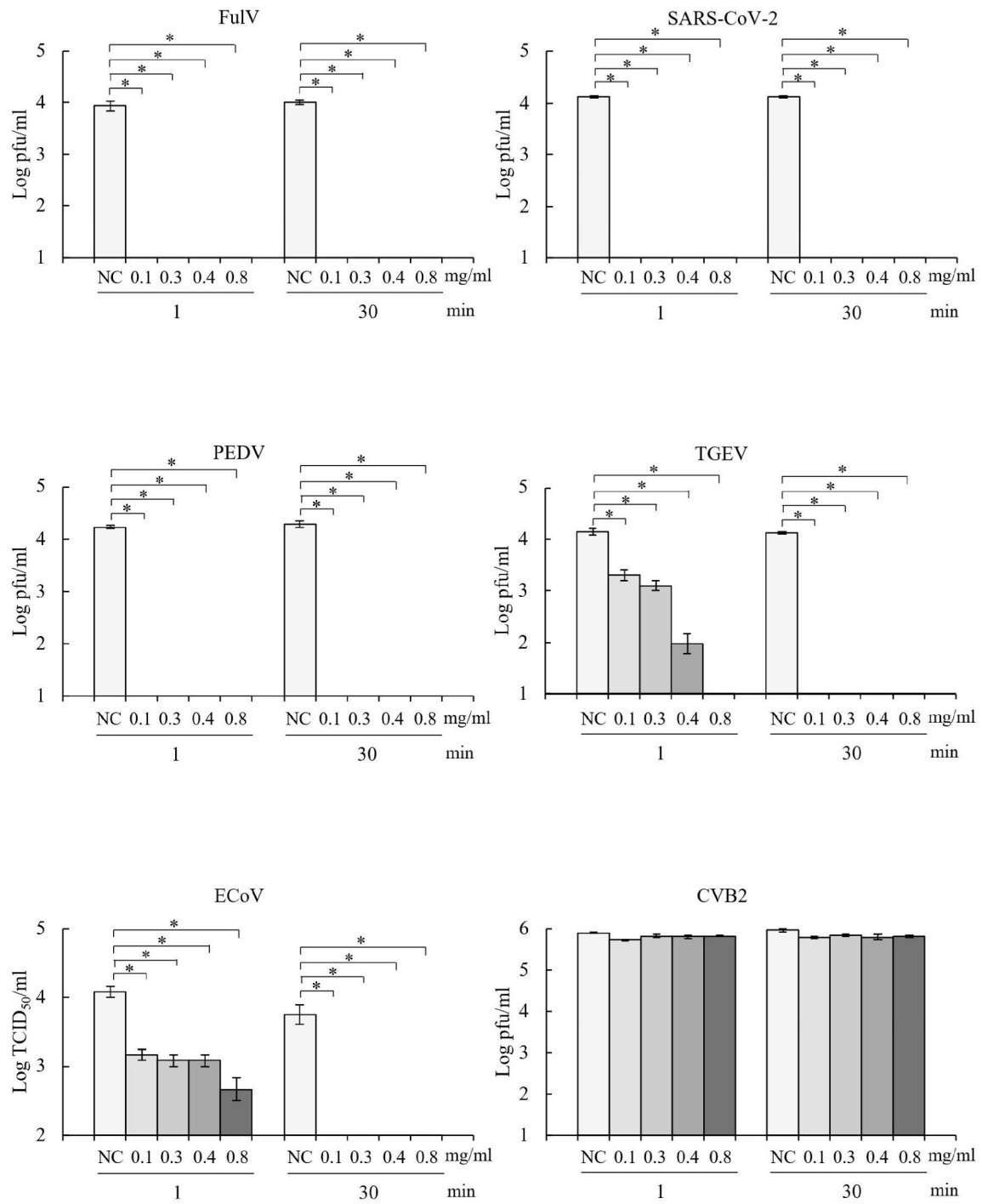


Fig.4



5.7. Tables

Table 1. Required dilutions of reaction samples containing weak acids and FaSSGF for virus titrations

virus	Cell lines used for virus titration	Required dilutions			
		Acetic acid	Oxalic acid	Citric acid	FaSSGF, surfactant
SARS-CoV-2	VeroE6/TMPRSS2	10^2	10^2	10^2	10^0
PEDV	Vero E6	10^2	10^2	10^2	10^0
TGEV	CPK	10^2	10^2	10^2	10^0
ECoV	HRT-18G	10^2	10^2	10^2	10^1
FuIV	MDCK	-	-	-	10^0
CVB2	Vero9013	-	-	-	10^0

5. General conclusion

In this dissertation, the virucidal efficacy of various common disinfectants was evaluated against both enveloped and non-enveloped viruses. The enveloped viruses tested included SARS-CoV-2, enteric coronaviruses, and feline calicivirus (FulV), while the non-enveloped viruses included coxsackievirus B2 (CVB2), human adenovirus type 56 (HAdV-D56), and feline calicivirus (FCV).

In CHAPTER 1, the viral inactivation efficacy of weak acids was assessed under different pH condition. The findings revealed that virucidal activity is determined not only by pH but also by the specific acid component. Interestingly, SARS-CoV-2, despite being an enveloped coronavirus, showed significant resistance to low pH conditions when exposed to oxalic acid. Furthermore, the results suggest that vinegars with acetic acid as its primary component, may serve as practical disinfectant against certain enveloped viruses, such as FulV in domestic environment.

These findings challenge the prevailing assumption that enveloped viruses are sensitive to low pH environment due to its lipid membrane. Therefore, it is essential to evaluate the virucidal spectrum of disinfectant across a broader range of both enveloped and non-enveloped viruses.

In CHAPTER 2, the authors explored the virucidal efficacy of 18 types of contact lens disinfectants containing various detergent components against the pathogenic viruses causing conjunctivitis, including of enveloped virus of SARS-CoV-2 and non-enveloped virus of HAdV-D56. Sodium hypochlorite- and hydrogen peroxide-based contact lens (CL) disinfectants reduced both viruses to below the detection limit. In contrast, salicylic acid and certain PHMB-based CL disinfectants effectively inactivated enveloped SARS-CoV-2 but not non-enveloped HAdV-D56, while other PHMB formulations, ϵ -polylysine, PQ-1 and alexidine hydrochloride did not exhibit efficient inactivation against either virus.

These results indicate that sodium hypochlorite- and hydrogen peroxide-based CL disinfectants have broad-spectrum virucidal activity against both enveloped and non-enveloped viruses. Given that HAdV-D56 is a major causative agent of conjunctivitis, CL disinfectants are recommended for daily contact lens sanitization in accordance with the manufacturer's protocol.

In CHAPTER 3, given the result as mentioned in CHAPTER 1, the ability of enveloped coronaviruses to survive in human gastric acid were examined. Despite of its lipid enveloped membrane, enteric coronaviruses including PEDV, TGEV and ECoV showed significant ability to withstand acidic conditions in the gastric environment. In contrast, SARS-CoV-2 was rapidly inactivated to below the detection limit.

These finding challenge the paradigm that enveloped viruses are generally vulnerable to acidic solutions due to its lipid membrane. These results revealed that gastrointestinal coronaviruses possess specific structural features within their envelope that confer resistance to low gastric pH, thereby enabling oral infection. The findings provide important clues for elucidating the mechanisms underlying oral infection by enteric coronaviruses.

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8. Abstract (English)

Common disinfectants are typically categorized by their antimicrobial spectrum for public sanitization. A prevailing assumption is that envelope viruses are more sensitive to acids and disinfectants due to lipid membrane, whereas non-enveloped viruses are more resistant because of proteinaceous capsid structure. However, this paradigm is based on limited experimental evidence. This study systematically evaluated enveloped viruses and non-enveloped pathogenic viruses under different acid types, disinfectant formulations, pH level, concentrations, and contact times.

These results are described in three chapters:

Chapter 1: pH-dependent virucidal effects of weak acids against pathogenic viruses.

Weak acid such as acetic, citric, oxalic acids are often considered to show virucidal effects through low pH condition. We assessed virucidal activity of these weak acids under varying pH conditions and common vinegars.

Weak acids were adjusted to pH 2, 4 and 6 and serially diluted from 1 M to 0.001 M. Enveloped viruses of Influenza A virus (FulV) and SARS-CoV-2, and non-enveloped virus of feline calicivirus (FCV) were exposed for up to 30 minutes. White distilled vinegar (WDV) and grain-flavored vinegar (GV) were also tested. Viral titers were quantified and compared in plaque-forming units (PFU/mL).

Acetic acid inactivated FulV at pH 4, whereas citric and oxalic acids did not. Both acetic and citric acids inactivated SARS-CoV-2 at pH 2, while oxalic acid was ineffective. All weak acids inactivated FCV at pH 2 but not at pH 4. Vinegar solutions reduced FulV titers within one minute and reduced SARS-CoV-2 titers within 30 minutes, with limited effect on FCV. Virucidal activity relied on pH and acid components for different viruses.

These results indicate that virucidal efficacy is influenced not only by pH but also by chemical properties. Acetic acid, particularly in vinegar form, shows promise as an a household sanitizer against enveloped viruses such as FulV. It highlighted the necessity for further evaluation on antiviral spectrum in disinfectants.

Chapter 2: Virucidal effects of contact lens disinfectants against enveloped and nonenveloped pathogenic viruses causing conjunctivitis.

Non-enveloped virus of Human Adenovirus D serotype 56 (HAdV-D56) and enveloped virus of SARS-CoV-2 are both reported as causative antigen for conjunctivitis. Contact Lens (CL) contamination could facilitate transmission for viral keratoconjunctivitis among CL users. This chapter evaluated the antiviral efficacy of commercial CL disinfectants.

Cytotoxicity was first assessed in A549 and Vero E6/TMPRSS2 cells to establish detection limits. Viruses were reacted to disinfectants according to manufacturer protocols. Viral titers were compared to untreated controls.

Sodium hypochlorite and hydrogen peroxide-based CL disinfectants reduced both SARS-CoV-2 and HAdV-D56 titers below detection limits. Salicylic acid and some polyhexamethylene biguanide (PHMB)-based solutions were effective only against SARS-CoV-2. Disinfectants with ϵ -polylysine, PQ-1, or alexidine hydrochloride showed minimal activity against either virus. Given the prevalence of adenoviruses causing viral conjunctivitis, daily disinfection using effective formulations is strongly recommended for CL users. These findings emphasize the need for targeted testing of ophthalmic disinfectants against viral pathogens beyond bacteria.

Chapter 3: Virucidal effects of contact lens disinfectants against enveloped and nonenveloped pathogenic viruses causing conjunctivitis

Despite of enveloped membrane, coronavirus such as PEDV, TGEV and EcoV cause severe enteric disease in swine and equine. This chapter investigated PEDV, TGEV, EcoV stability in low pH in simulated gastric conditions.

PEDV, TGEV, ECoV, SARS-CoV-2, CVB2 and FulV were exposed to weak acids (1M, pH 2) and simulated gastric fasted-state simulated gastric fluid (FaSSGF) (pH 1.6) for up to 90 mins.

Citric and acetic acids (1 M, pH 2) significantly inactivated PEDV, TGEV, and EcoV. Oxalic acid showed limited efficacy, similar to SARS-CoV-2. Interestingly, SARS-CoV-2 was rapidly inactivated in FaSSGF (pH 1.6), while PEDV, TGEV, and ECoV remained their infectivity for up to 90 mins. Surfactants in FaSSGF did not affect their stability, but pepsin in FaSSGF (pH 1.6) exhibited moderate virucidal activity.

These findings indicate that animal enteric coronaviruses possess unique structural or biochemical adaptations enabling survival in acidic conditions. For neonates with underdeveloped gastric lumen including of low pepsin, high pH level after meal., leading to oral transmission.

Above all, this study demonstrated that virucidal effect depends on the chemical composition, pH, and virus characteristics rather than virus enveloped membrane. Enveloped coronaviruses may possess acid-tolerance membrane structure. These insights highlight the need for further morphological and mechanistic studies to inform effective disinfection strategies in clinical and domestic settings.