IN VITRO ANTI-VIRAL ACTIVITY OF HEXETIDINE (BACTIDOL®) ORAL MOUTHWASH AGAINST HUMAN CORONAVIRUS OC43 AND INFLUENZA A (H1N1) VIRUS

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ABSTRACT

Mouthwashes are used to decrease oral cavity microbial load due to their antiseptic properties. Hexetidine is a broad-spectrum antiseptic used for minor infections of mucous membranes, and in particular as a 0.1% mouthwash for local infections and oral hygiene.

This study determined the anti-viral activity of the mouthwash hexetidine (Bactidol®), specifically in reducing viral concentration of Human Coronavirus OC43 (HCoV OC43; ATCC® VR-1558™) and Influenza A virus (IAV H1N1; clinical strain) in Vero 6 and MDCK cell cultures respectively, using in-vitro suspension assay (ASTM E-1052-11) designed to evaluate virucidal property of microbicides like hexetidine.

Study results indicated that hexetidine was able to reduce infectivity of HCoV OC43 and IAV H1N1 at 25%, 50% and 100% concentrations by more than 80% at 15- and 30-seconds exposure times. One hundred percent (100%) concentration of hexetidine was found to be cytotoxic to MDCK cell line used for IAV H1N1 propagation. Hexetidine-treated cell lines achieved >80% survival rate for MDCK and Vero E6 at a contact time of 15 seconds and 30 seconds (which are the approximate times of gargling with hexetidine mouthwash).

The anti-viral activity of hexetidine mouthwash against other more virulent or pathogenic coronaviruses like SARS-CoV-2 can be explored further.

KEYWORDS: Hexetidine, mouthwash, human coronavirus, influenza A virus

INTRODUCTION

Mouthwashes have been widely used in oral hygiene. Most of the mouthwashes are used to decrease oral cavity microbial load due to their anti-septic properties and have been prescribed in dentistry. The active ingredients of mouthwashes, among others are hexetidine, benzydamine hydrochloride, and povidone iodine.

Hexetidine belongs to the group of pyrimidine derivatives. It is a broad-spectrum antiseptic, active *in vitro* and *in vivo* against Gram-positive and Gram-negative bacteria. It is used for minor infections of mucous membranes, and in particular as a 0.1% mouthwash for local infections and oral hygiene. Pharmacological evidence indicated that the primary mode of action is due to its interference with vital metabolic processes necessary for the growth of microorganisms [1].

Povidone iodine is an iodophore that is used as a disinfectant and antiseptic. Iodophores are loose complexes of iodine and carrier polymers. Solutions of povidone-iodine gradually release iodine to exert an effect against bacteria, fungi, viruses, protozoa, cysts, and spores. A 1% mouthwash has been used for oral infections including candidiasis [1].

Antimicrobial efficacy may be measured directly by the effect of certain compounds on the growth of microorganisms *in vitro*, or indirectly by studying their effect on certain health conditions that are caused or affected by microorganisms. Some of these conditions are halitosis, plaque, gingivitis and periodontitis. [1]

From the literature review above [2], hexetidine and povidone iodine generally had good efficacy against the bacterial flora of the oral cavity, with hexetidine being slightly superior between the two. In addition, hexetidine lost only approximately 25% of its antimicrobial action after 60 minutes while povidone iodine lost almost 40% of its antimicrobial efficacy after 10 minutes [3].

The sustained antimicrobial efficacy of hexetidine was also noted in another study [4]; however, it was noted that the duration was shorter than for chlorhexidine [3,4]. Hexetidine was also found to have good efficacy against plaque and gingivitis [5,6] but was again inferior to chlorhexidine [7].

Four other studies found that the antimicrobial action of povidone iodine had a short duration [3,11-13]. Some studies, however, found povidone iodine had higher antimicrobial efficacy than chlorhexidine [14,15]. Povidone iodine was also noted to reduce *Streptococcus mutans* count although not to the same degree as chlorhexidine [16]. It was more effective in alleviating mucositis than chlorhexidine [8] and was as effective as chlorhexidine for plaque and gingivitis [17].

The literature review above [2] concluded based on the *in vitro*, *in vivo* and clinical studies discussed, which used both direct and indirect methods to measure antimicrobial efficacy, that hexetidine, chlorhexidine and povidone-iodine were effective against oral microbial flora and differ primarily in the duration of their antimicrobial action.

Zoonotic coronaviruses were discovered in the 1960s and pathogenic human coronaviruses were discovered in 2002. Currently, there are now seven human coronaviruses which include the SARS-CoV-2. Most of these human coronaviruses cause mild diseases, and these include the HCoVs OC43, 229E, NL63 and HKU1. The more virulent species are SARS-CoV, MERS-CoV and SARS-CoV-2. Coronaviruses are positive sense, single stranded RNA virus, spherical and enveloped with club-shaped spikes on the surface looking like the solar corona. The four coronaviruses genera are α, β, γ, δ. The human α-CoV are HCoV-229E and NL63, while the human β-CoV are MERS-CoV, SARS-CoV, HCoV OC43 and HCoV-HKU1. SARS-CoV-2 and HCoV OC43 are both β-CoV.[18]

This study determined the anti-viral activity of hexetidine (Bactidol®), specifically in reducing viral concentration of Human Coronavirus (HCoV OC43) and Influenza A virus (IAV H1N1) infected cell cultures by demonstrating its Tissue Culture Infective Dose or TCID50/ml in 25%, 50% and 100% aqueous hexetidine concentration at 15- and 30-seconds exposure times (which are the approximate times of gargling with Bactidol® mouthwash).

MATERIALS and METHODS

The study was done from 10 September 2020 to 03 November 2020. The test method used was according to the American Society for Testing and Materials International - ASTM E-1052-11 Standard test method, which assess the activity of microbicides against viruses in suspension (2011) [19].

Tissue culture cells. Madin Darby Canine Kidney (MDCK) cells from WHO Collaborating Centre for Reference and Research on Influenza, Australia, and African green monkey kidney Vero E6 cells from the Department of Virology, Tohoku University Graduate School of Medicine, Sendai, Japan were used. These cell lines are permissive and susceptible for the test viruses IAV H1N1 and HCoV OC43, respectively. These cultures were maintained in tissue culture media with additives.

Test viruses. Influenza A virus H1N1 (IAV H1N1), came from a clinical sample from Influenza Surveillance, were grown in MDCK cells, confirmed by Hemagglutination Inhibition and RT-PCR. Human Coronavirus (HCoV FR-302), Strain OC43 (ATCC®VR-1558™) was purchased from American Type Culture Collection (ATCC). These are the common respiratory viruses that can cause sore throat and are found in the oral cavity; HCoV is a β-CoV similar to SARS-CoV-2. These viruses were grown on viral culture media with additives (L-glutamine, Penicillin-Streptomycin).

Antiviral agents. Hexetidine (Bactidol®) at 100% (undiluted), aqueous 50% and 25% concentrations were used. 70% ethyl alcohol served as a positive control.

The experimental conditions were: test temperature at 37°C; neutralizer used, Minimum Essential Medium (Gibco brand, Catalogue No. 11700-077) with 2% Fetal Bovine Serum (heat inactivated, Gibco Brand, Catalogue No. 10500-064); incubation time for IAV H1N1 - 5 days, HCoV OC43 - 3 days ; and incubation temperature for IAV H1N1 - 35°C, HCoV OC43 – 37°C.

The method used was an *in-vitro* suspension assay designed to evaluate virucidal property of the product against IAV H1N1 and HCoV OC43. Presence of infective virus was determined via monitoring of cytopathic effect (CPE) on the appropriate cell line. A 0.2 ml suspension of virus was exposed to 1.8mI per specified concentration of anti-viral test agents. At each specified exposure time, an aliquot was removed and neutralized by serial dilution and assayed for presence of virus. Virus controls, cytotoxicity controls, and neutralization controls were assayed in parallel.

After incubation, virus infected cell cultures in wells were identified through their characteristic CPE, rounding of cells (MDCK) and vacuolization of the cytoplasm and sloughing of cells (Vero E6). Recorded results were used to calculate infecting activity (TCID50) through Spearman-Karber method [20]. Percent reduction and log reduction were subsequently computed.

All tests were done at the Virology Department of the Research Institute for Tropical Medicine (RITM), Department of Health. The Institutional Review Board (IRB) of RITM was accordingly informed of this basic laboratory study involving viral cell cultures and does not require to undergo review as per regulations on studies involving animals or humans as test subjects.

RESULTS

The results of the ASTM E-1052-11 assay, hexetidine (Bactidol®) against IAV H1N1 (clinical strain) and HCoV OC43 (ATCC® VR-1558™) are shown in Table 1. Hexetidine (Bactidol®) at 25%, 50% and 100% aqueous concentrations against HCoV OC43 (ATCC® VR-1558™) showed from 94.38 % to 99.68% infectivity reduction at 15 seconds contact time, and from 94.38% to 99% infectivity reduction at 30 seconds contact time.

Hexetidine (Bactidol®) at 25% and 50% aqueous concentrations against IAV H1N1 (clinical strain) showed 82.22% and 94.38% infectivity reduction at 15 seconds contact time, and from 94.38% and 99% infectivity reduction at 30 seconds contact time, at respective concentrations.

However, one hundred percent (100%) concentration of hexetidine (Bactidol®) was found to be cytotoxic to the MDCK cell line used for IAV H1N1 propagation.

Table 1. Computed Log10 reduction and percent (%) reduction values for IAV H1N1 (clinical strain) and HCoV OC43 (ATCC® VR-1558™) challenged with three concentrations of hexetidine (Bactidol®) at 15- and 30- seconds exposure times.

Test Virus	**Bactidol® Concentration (%)**	Exposure at 15 seconds		Exposure at 30 seconds	
		Log10 Reduction	% Reduction	Log10 Reduction	% Reduction
Human Coronavirus OC43	100	1.98	99.68	1.48	99.00
	50	0.73	94.38	0.73	94.38
	25	1.73	99.44	1.23	98.22
Influenza A Virus H1N1	100	*	*	*	*
	50	0.5	94.38	2.00	99.00
	25	1.73	82.22	1.23	94.38

Note: * Bactidol at 100% (undiluted) is cytotoxic to MDCK cells.

The following figures were taken during the study, Hexetidine (Bactidol®) mouthwash showed inhibition of the virus induced CPE in the infected MDCK and Vero 6 cells, Figure 1. letters A and C, respectively.

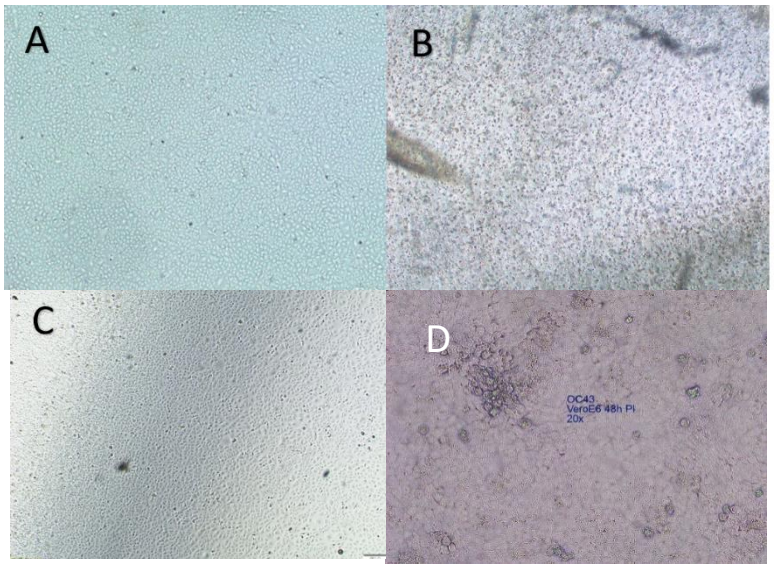

Figure 1. **(A)** confluent MDCK monolayer without IAV CPE, **(B)** MDCK monolayer infected with IAV exhibited rounding of cells cytopathic effect; (**C**) confluent Vero E6 monolayer without HCoV OC43 CPE; (**D**) Vero E6

monolayer infected with HCoV OC43 exhibited vacuolization of the cytoplasm and sloughing of cells cytopathic effect

DISCUSSION

This study showed that hexetidine (Bactidol®) was able to reduce infectivity of HCoV OC43 (ATCC® VR-1558™) and IAV H1N1 at 25%, 50% and 100% concentrations. These hexetidine concentrations reduced by more than 94% to 99% the infectivity of Human Coronavirus OC43 at 15 and 30 seconds exposure times.

For Influenza A Virus H1N1 (clinical strain) showed that at lower, 25% hexetidine concentration 82.22% and 94.38% infectivity reduction was found at 15 and 30 seconds contact time, respectively. At 50% concentration, 94.38% and 99% infectivity reduction at 15 and 30 seconds contact time, respectively. Undiluted hexetidine (Bactidol®), 100% concentration was cytotoxic to the MDCK cell line used for Influenza A H1N1 propagation.

Hexetidine mouthwash inhibited influenza A virus (IAV) and human coronavirus (HCoV OC43). Thus, hexetidine (Bactidol®) in 25%, 50%, and 100% concentrations can be used as an effective mouthwash over 15 to 30 seconds to get rid of HCoV OC43 and IAV H1N1 on the oral mucosal surfaces.

The study of Deryabin P.G., et al., "Analysis of Antiviral Properties of Hexoral *In Vitro* against Some Viruses that Cause Acute Respiratory Infections and Herpes" has shown similar results. Hexoral® (with Hexetidine as the active ingredient) has shown to have an antiviral property against viruses causing human respiratory tract infections and herpes virus. [21] Exposure to Hexoral® and Hexetidine alone for 30 secs. was able to attenuate the infectivity of Influenza Virus A/H5N1, pandemic Influenza Virus A/H1N1pdm, Respiratory Syncytial Virus, and Herpes Simplex Virus type 1 by 100 or more times. [21]

Antiseptic mouthwashes have been widely used as a standard measure before routine dental treatment, especially pre-operatively [22,23]. These are widely used solutions for rinsing the mouth due to their ability to reduce the number of microorganisms in the oral cavity and colony-forming units in dental aerosols [24]. The use of mouthwashes and gargles were deemed to be relatively safe, with only minor adverse effects with long-term use [25].

Vergara-Buenaventura A and Castro-Ruiz C (2020) suggested the use of pre-procedural mouthwashes in dental practice to reduce SARS-CoV-2 viral load from dental procedures and to reduce the cross-infection risk while treating patients during the pandemic (26).

The SARS-CoV-2 spike protein in the membrane envelope is a typical structure of coronaviruses [26- 28]. The spike protein interacts with the angiotensin-converting enzyme 2 (ACE2) receptors of the host cells enabling the virus to enter the cells [29]. Different tissue cells, including those of the mucosal tissues, gingiva, periodontal pockets, tongue and salivary glands present possible infection routes as these have membranes bound to ACE2 [29-34]. Such oral tissues

are sources from which SARS-CoV-2 transmission may occur during dental care, talking, coughing, and sneezing (35,36).

The American Dental Association [37] and the Center for Disease Control and Prevention [38] have recommended the use of pre-procedural mouthwashes before oral procedures, despite no clinical evidence that the use of mouthwashes could prevent SARS-CoV-2 transmission. [26]

In the Philippines, among the active agents used in commercially available mouthwashes, only povidone-iodine had been shown to have antiviral activity *in vitro* against SARSCoV-2 [39].

This *in vitro* study showed that hexetidine, even at diluted concentrations reduced the infectivity of the two oral virus strains, HCoV OC43 and Influenza A virus H1N1 when used for 15 and 30 seconds. The anti-viral activity of hexetidine mouthwash against the other more virulent members of the Coronavirus Family, SARS-CoV-2 can be explored using the methods used in this *in vitro* study. Clinical studies can also follow, to evaluate the effectiveness of antiseptic mouthwashes like hexetidine on SARS-CoV-2, the subjects may be with asymptomatic or mildly infected individuals. The future study will determine whether hexetidine (Bactidol®) can lessen viral load or shorten the duration of viral carriage.

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