STUDY OF THE ANTIMICROBIAL ACTIVITY OF AN ORAL GEL: KLIRICH

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ABSTRACT

The oral cavity can be affected by multiple conditions (dental plaque, bleeding, inflammations, periodontal diseases such as gingivitis, periodontitis and other abscesses, oral aphthae and other oral ulcerations).

The etiology of these conditions is mainly bacterial (*Prevotella melaninogenica*, *Streptococcus sanguinis, Porphyromonas gingivalis, Actinomyces naeslundii, Actinomyces israelii, Aggregatibacter actinomycetmcomitans etc.)*, fungal *(Candida albicans, etc.)* or viral (*Herpes Simplex*, etc.).

The *in vitro* antimicrobial efficacy of the oral gel named "KLIRICH" was demonstrated on these microorganisms of interest and is the main subject of this publication.

KLIRICH is a class I medical device, whose efficacy and clinical tolerance have been demonstrated among patients suffering from inflammatory gingivitis with or without associated symptoms. The results of this present study show that KLIRICH is proven to have antimicrobial properties against microorganism of interest regarding oral inflammation, which can moreover place it as a preventive product.

INTRODUCTION

The mouth is a moist environment which is the seat of a complex ecosystem essentially made up of commensal microorganisms (bacteria, mycoplasmas, protozoa, viruses).

The oral cavity is accessible to different types of microorganisms (coming from hand, dust or water). The oral flora is therefore extremely varied in terms of both quantity and quality, but the predominant microorganisms remain the bacteria.

This natural cavity constitutes one of the most colonized-parts of the human organism.

The abundance and virulence vary according to individuals, their general condition and the local conditions.

The surfaces of the lips, cheeks, palate, tongue, gingiva, and teeth are covered with a very hydrophilic film composed of mucins which greatly favours bacterial adherence.

The issue is that commensal species can become pathogenic stemming from various modifications including an imbalance of the oral ecosystem.

We focused on gingivitis, a periodontal disease characterized by a gingiva inflammation, with or without bleeding. Gingivitis is mainly due to the presence of dental plaque (a sticky film mainly composed of bacteria, but also salivary proteins, sugars and acids, which accumulate on the teeth). After having multiplied, the pathogens produce toxins which intensify the disease and the associated symptoms.

This oral inflammation is frequent and can develop into a chronic systemic disease.

After a preliminary market study, it was observed that there is currently no truly effective and satisfactory solution for the treatment of inflammatory conditions of the oral cavity. Moreover no formulation is likely to really adhere to the affected mucosa to relieve it, but also to soothe the oral sphere following a dental care intervention.

In order to meet the medical needs, a product was created and patented (publication EP2954902 A1), the invention bears the name "KLIRICH". This oral gel, classified by regulation as class I medical device, makes it possible to fight against the inflammation due to periodontal diseases. Its efficacy and tolerance ~~is~~ are proven thanks to a prospective randomized clinical study *versus* placebo, when used on gingiva by massage.

Regarding the frequent microbial etiology of the highlighted pathologies, it was interesting to study the *in vitro* antimicrobial efficacy of this product. Tests were therefore conducted on the following microorganisms of oral interest:
Prevotella melaninogenica, *Streptococcus sanguinis, Porphyromonas gingivalis, Actinomyces naeslundii, Actinomyces israelii, Aggregatibacter actinomycetmcomitans, Candida albicans, Herpes Simplex*.

PRODUCT PRESENTATION

COMPOSITION AND GALENICS

KLIRICH is a gel composed of pleiotropic substances ensuring its optimal mucoadhesivity in the aqueous oral cavity. Thus, the gel protects the gum, impregnates it by massage and so decreases the pain and the inflammation as demonstrated by the results of the randomized clinical study previously quoted.

The main aim of this publication is to demonstrate the antimicrobial properties of the gel on the microorganisms of interest in the inflammation of the inflammatory gingival pathologies.

PROPERTIES

KLIRICH is a gel which is effective on inflamed oral cavities of adults (gingivitis or associated symptoms such as bleeding, gingival recessions, periodontal pockets).

An antimicrobial activity exists and has been demonstrated *in vitro* on strains involved in these various pathologies but also subjacently *in vivo* on a target clinical population affected by gingivitis

MATERIALS AND METHODS

- Standard methods :

The test methods for evaluating the *in vitro* antimicrobial efficacy of KLIRICH relate to chemical antiseptics and disinfectants and are European standards.
These tests use standardized strains in collection which are supposed to represent the whole microbial world, by classes of microorganisms and allow to demonstrate the potential antimicrobial activity in suspension or on carriers.
The aim was therefore to show the antimicrobial efficacy of KLIRICH against pathogens of the oral sphere according to the methodology of the following standards:

* ISO 11930[(1)] - Evaluation of the antimicrobial protection of a cosmetic product
* EN 13727[(2)] - Quantitative suspension test for the evaluation of bactericidal activity in the medical area

* EN 13624[3] - Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity in the medical area
* EN 14561[4] - Quantitative carrier test for the evaluation of bactericidal activity for instruments used in the medical area
* EN 14562[5] - Quantitative carrier test for the evaluation of fungicidal or yeasticidal activity for instruments used in the medical area
* EN 14476[6] - Quantitative suspension test for the evaluation of virucidal activity in the medical area - Test method and requirements (phase 2, stage 1)

- Experimental conditions:

Due to salivation, the gel has a short lifetime in the mouth, after application.

In order to approximate real use conditions of the product as much as possible, the tests were performed by observing a very short contact time (from 30 to 60 seconds).

Furthermore, an interfering substance containing 3ml/l of sheep erythrocytes intended to mimic the presence of blood were set up.

All of these *in vitro* tests were performed on a not totally gelled product for reasons link to technical feasibility.

- Strain collection and culture media:

The flora of a periodontal pocket is characterized by a high proportion of anaerobic microorganisms (90%) the majority of which are Gram negative bacteria.

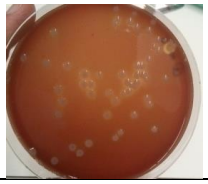
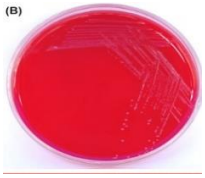
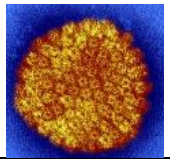
The study concerned different strains able to interfere in the oral cavity in case of periodontal pathologies[14].

These mic microorganisms come from cell cultures of the American Type Culture Collection (ATCC) and of the German collection (DSMZ; Deutsche Sammlung von Mikrorganismen und Zellkulturen).

The Herpes virus was also tested, the tests were conducted on the strain *Herpes simplex* type 1 cultivated on VERO cells.

- Characteristics of the different bacterial strains used:

Strains and collection number	Characteristics and culture conditions	Associated pathologies
Porphyromonas gingivalis DSM 20709 	- Mobile, anaerobic gram - bacillus - Periodontopathogen - Columbia agar with sheep blood (5%) at 37°C	Periodontal diseases and abscesses
Prevotella melaninogenica DSM 7089 	- Mobile anaerobic Gram - bacillus - Oral pathogen in association with other microorganisms - Columbia agar with sheep blood (5%) at 37°C	Periodontal diseases Periodontal abscesses (Important role in the pathogenesis of the disease)
Streptococcus mutans DSM 20523 	- Non mobile, aerobic Gram + coccus - Commensal flora of the oral cavity - TSA agar at 37°c	Dental plaque and caries Weakening of the gingiva and of the dental enamel. Tendency to produce a polysaccharide biofilm.

Streptococcus sanguinis ATCC 10556 	- Non mobile, facultatively anaerobic Gram + coccus - Oral pathogen - Columbia agar or TSA with sheep blood (5%) atmosphere 5% CO2 at 37°c	Caries and weakening of the gingiva
Actinomyces naeslundii DSM 43013 	- Non mobile, Gram + bacillus, facultatively anaerobic - Oral pathogen - Columbia agar with sheep blood 10%) at 37°C	Dental plaque Periodontal diseases
Actinomyces israelii DSM 43420 	- Non mobile, Gram + bacillus, anaerobic - Oral pathogen - Columbia agar with sheep blood 10%) at 37°C	Dental plaque Periodontal diseases
Aggregatibacter actinomycetmcomitans DS 8324 	- Gram - bacillus, anaerobic - Columbia agar with sheep blood 10%) at 37°C	Periodontal diseases
Candida albicans DSM 1386 	- Aerobic yeast - saprophytic commensal microorganism - MEA agar at 30°C	Candidiasis commonly known as oral thrush.
Herpes virus type 1 ATCC VR-260 	- Enveloped DNA virus - Culture on VERO cells, 5% of CO2 atmosphere at 37°C	Oral Herpes

- Composition of the culture media (broth and agar)

TSB

	Grams/liter
Pancreatic digestion of casein	17.0
Soybean peptone	3.00
Sodium chloride	5.00
Glucose	2.50
Dipotassium phosphate	2.50
Distilled water	QS 1L

MEA (pH = 5.4)

	Grams/liter
MALT extract	30.0
Soybean peptone	3.00
Agar-agar	15.0

TSA (pH = 7.3)

	Grams/liter
Tryptone	15.0
Soybean peptone	3.00
Sodium chloride	5.00
Agar-agar	15.0

Columbia with blood (pH = 7.3)

	Grams/liter
Peptone of casein	10.0
Peptone of meat	5.00
Peptone of heart	3.00
Yeast extract	5.00
Corn starch	1.00
Sodium chloride	5.00
Agar-agar	13.5
Defibrined Sheep Blood	50 ml
Purified water	QS 1L

- Inclusion in the collection

The bacteria and yeast are received freeze-dried. During their culturing, they are rehydrated under sterile conditions in nutrient broth, and then inoculated on agar.
Macroscopic (aspect of the colonies, oxidase and catalase tests) and microscopic (morphology, Gram staining) identifications are performed in order to control their purity and identity.
A cryoprotective solution makes it possible to keep them in the freezer at -70°C.

- *Cryoprotective solution*

	Grams/liter
Meat extract	3.00
Peptone	5.00
Glycerol	150.0
Purified water	QS 1L

Specific atmospheres are required for the culture of some microorganisms:

For *P. melaninogenica* and *P. gingivalis*, a jar incubation with specific sachets is required to achieve a complete anaerobiosis.
For *S. sanguinis and Herpes simplex* type 1 virus, a modified atmosphere at 5% of CO2 is used for their incubation.

- Mother culture:

For *S. mutans*, the mother cultures are made on TSA agars tilted and incubated at 37°C for 24h.
For *S. sanguinis, P. gingivalis* and *P. melaninogenica*, *Actinomyces naeslundii, Actinomyces israelii, Aggregatibacter actinomycetmcomitans* the platings are performed on sheep blood (5% or 10%) Columbia agars and the incubation time is from 48 to 72h in a jar.
The protocol for the yeast *C. abicans* is similar but the incubation is performed at 30°C for 48h on MEA agars. After incubation, each mother culture (R0) is kept in the refrigerator at 4°C.

- Working culture

The working culture is prepared by taking with a loop a sample of the mother culture to inoculate a new sterile culture medium (subculture R1). After incubation, the R1 is then subcultured to obtain the working culture R2. The R2 culture is the one to be used for the tests. It should be noted that a R3 subculture can be made from the R2 culture in case of need, but no additional subculture should be prepared after this one. To achieve the cell concentration required for the tests, a calibration range is made. For this, suspensions of different concentrations are prepared from R2 subcultures and tryptone salt (TS). The corresponding optical densities (OD) are read on the spectrophotometer at 620 nm, then a count on dishes is performed. Hence, each absorbance is related to a concentration of microorganisms in Colony Forming Unit per milliliter (UFC/ml).

During a test, the inoculum is adjusted with tryptone salt (TS) until reaching an OD corresponding to a required cell concentration.

TS diluent (pH = 7.5)

	Grams/liter
Tryptone, pancreatic digestate of casein	1.00
Sodium chloride	8.50

- Description of the standards performed

- *Antimicrobial protection: International standard ISO 11930*

The standard ISO 11930[1] commonly called challenge test, enables the *"evaluation of the antimicrobial protection of a cosmetic product".* Classically, five strains are tested: the three bacteria *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus*; the filamentous fungus *Aspergillus brasiliensis* (formerly *Aspergillus niger*) and the yeast *C. albicans*.

The principle of this test is to put in contact a calibrated suspension of microorganisms (from $1x10^7$ to $1x10^8$ UFC/ml for bacteria and $1x10^6$ to $1x10^7$ UFC/ml for the yeast and the mould) with the product to be tested. The inoculated samples are kept in the dark at ambient temperature.

After 7, 14, and 28 days of contact, samples of product inoculated with each microorganism are taken, then neutralized before being inoculated on appropriate culture media. A logarithmic reduction is calculated for each strain and compared with the requirements.

The standard states that a logarithmic reduction greater than or equal to 3 for bacteria, greater than or equal to 1 for the yeast and the mould, corresponds to a good preservation of the cosmetic product. Furthermore, no increase of microbial cells should appear after 14 and 28 days.

In parallel to the inoculation of the product at T0, two controls are performed. A first neutralization control checks that the neutralizer used is effective in stopping the antimicrobial activity of the tested product, and a second control of non-toxicity ensures that this neutralizer does not impact the survival of the microorganisms.

- *Evaluation of antimicrobial efficacy: European standards*

The phase 1 standards make it possible to evaluate the basic activity regardless of the use of the product: qualification of the antimicrobial activity.

The phase 2 standards follow the methodology of the phase 1 by integrating an interfering substance (IS) to simulate the condition of the surface to be disinfected under real conditions. Two interfering substances can be used: the clean condition is a surface which has been cleaned prior to disinfection, whereas the dirty condition is a surface which should be disinfected in the presence of soiling.

These standards of the phase 2 are sub-divided into two stages, a stage 1 testing the product in suspension and a stage 2 to test the product on a representative surface (stainless steel, polycarbonate, silicone, etc.).

- *EN 13624 and EN 13727 standards*

The principle of the EN 13624[2] and EN 13727[3] standards is to put in contact the product to be tested with a calibrated suspension of microorganisms. In the case of the oral gel, an interfering substance (IS) composed of TS and erythrocytes is added. This IS makes it possible to simulate a bleeding of the oral mucosa frequently occurring during infections, in order to approximate real conditions of use as much as possible.

After a defined contact time, a part of this mixture is transferred into some neutralizer to stop the antimicrobial activity of the tested product at the end of the contact time. Following the neutralization, an inoculation in duplicate is performed on an agar medium appropriate for the tested microorganism. The results are counted after the required incubation time.

These standards can be performed according to two different methods: neutralization dilution or membrane filtration. This last method is used when no effective neutralizer is found. Membrane filtration makes it possible to eliminate the product at the end of the contact time by retaining the microorganisms, since the diameter of the pores is 0.45µm. Hence the product is eliminated by flow, which replaces the product neutralization stage.

- *EN 14561 and 14562 standards*

To test the antimicrobial activity of the product on carriers in clinical settings, the EN 14561[4] and EN 14562[5] standards were performed.

These tests consist in inoculating a mixture of a microbial suspension and an interfering substance (erythrocytes and TS) on a test surface (glass or stainless steel 316 L are the appropriate carriers). This surface is then oven dried at 37°C for a maximum 60 minutes. After drying, the carrier is immersed in the product to be tested for a defined contact time. After a neutralization stage, inoculations are performed in duplicate on Petri dishes.

In all the standards presented above, three controls are performed in parallel in order to validate the tests:

- the first makes it possible to check the survival of the microorganisms under the experimental conditions of the test.
- the second ensures that the neutralizer has no lethal effect on the tested microorganisms.
- the third control makes it possible to validate that the neutralizer used in the test enables the inhibition of the potential antimicrobial activity contained in the product.

For all these controls, the number of survivors is counted on dishes. The counts must be equal to or greater than 50% of the validation suspension count to consider that the actual tests can be exploited.

In the carrier tests, an additional control is performed. The water control is a recovery control which makes it possible to control that the drying of the carriers has no significant influence on the survival of the inocula. They should be visually dry to simulate the adherence of the microorganisms on the surface as best as possible, but not too much either so that only the tested product is evaluated. The drying must not be lethal.

- *EN 14476 standard*

As for the EN 13624[2] and EN 13727[3] standards, the principle of the EN 14476[6] standard is to put a sample of the product to test in contact with a calibrated suspension, here a virus. In the case of the oral gel, an interfering substance (IS) composed of TS and erythrocytes is added. This IS makes it possible to simulate a bleeding of the oral mucosa frequently occurring during infections, in order to approximate real conditions of use as much as possible.

After the defined time of contact, an aliquot of this mixture is transferred into cold diluent to stop the virucidal activity of the tested product then this new mixture is put into cell cultures. The infectivity tests were carried out by cytopathic effect titration (suspension cell method).

After incubation, the infectious titers are calculated according to Spearman and Kärber and are evaluated.

The virus infectivity reduction is calculated and corresponds to the difference between the virus titers (viral control), expressed in lg, before and after the treatment with the product.

- Test validation

During the testing on an antiseptic or disinfectant product, a contact time is defined corresponding to the product's action time claimed by the manufacturer.

During the tests except the virucidia, a neutralizer's solution is used to stop the antimicrobial activity at the time set and check the actual antimicrobial activity of the product.

Neutralizer

The neutralization can be performed by neutralization dilution or by membrane filtration.

The European standards recommend different neutralizers according to the active substances present in the product to be tested. A polyvalent neutralizer (containing saponin, polysorbate 80, and lecithin) is used as a first choice for any type of product.

In case of failure, a second specific neutralizer can be used (D/E neutralizing broth (39g/I) known for neutralizing antimicrobial agents such as quaternary ammonium compounds, fatty amines, and amphoteric compounds.

In order to succeed in neutralizing KLIRICH, two additional substances had to be introduced: sodium dodecylbenzenesulfonate (NaDBS) and sodium thiosulfate.

Rinsing liquid

During the membrane filtration tests, the product passes through the nitrocellulose membrane (pores with a 0.45µm diameter) which enables the neutralization of the product. This method requires a liquid for rinsing the membrane. The rinsing liquid recommended in the standards contains tryptone, sodium chloride, as well as polysorbate. In this study, it was completed with 1% of NaDBS in order to allow for a more effective rinsing of the membranes.

Neutralizers and rinsing liquid composition

Polyvalent neutralizer

	Grams/liter
Saponin	30.0
Polysorbate 80	30.0
Soybean lecithin	3.00
Sodium thiosulphate	5.00
L-histidine	1.00
Demineralized Water	QS 1L

Specific neutralizer (pH = 7.6)

	Grams/liter
Tryptone	5.00
Yeast extract	2.50
Sodium bisulfite	2.50
Glucose	10.0
Soybean lecithin	7.00
Polysorbate 80	5.00
Sodium thioglycolate	1.00
Sodium thiosulphate	6.00
Bromocresol purple	0.02

Specific neutralizer + 1 to 5% of NaDBS

	Grams/liter
Tryptone	5.00
Yeast extract	2.50
Sodium bisulfite	2.50
Glucose	10.0
Soybean lecithin	7.00
Polysorbate 80	5.00
Sodium thioglycolate	1.00
Sodium thiosulphate	6.00
Bromocresol purple	0.02
Sodium dodecylbenzenesulphonate	From 1.00 for 1% to 5.00 pour 5%

Specific neutralizer + 10 to 20g/l of sodium thiosulphate

	Grams/liter
Tryptone	5.00
Yeast extract	2.50
Sodium bisulfite	2.50
Glucose	10.0
Soybean lecithin	7.00
Polysorbate 80	5.00

Sodium thioglycolate	From 10.0 to 20.0
Sodium thiosulphate	6.00
Bromocresol purple	0.02

Rinsing liquid

	Grams/liter
Sodium chloride	5.00
Tryptone	2.50
Sodium dodecylbenzenesulphonate	1.00 for 1% - 2.00 for 2%
Polysorbate 80	5.00

With the exception of *P. melaninogenica, Actinomyces naeslundii, Actinomyces israelii, Aggregatibacter actinomycetmcomitans* for which a membrane filtration had to be performed, all the strains tested followed the neutralization dilution method.

RESULTS

CHALLENGE TESTS

Since the KLIRICH product is a treatment intended to come into contact with consumers (or patients), an optimized preservation is necessary to guarantee its microbiological safety.

The challenge test ISO 11930 performed on the oral gel showed that the product is protected against any external contamination which could occur during its use.

In this test, the product samples are inoculated at 10^6 UCF/ml. This test was performed with the polyvalent neutralizer. For all the strains except *S. aureus*, the polyvalent neutralizer proved to be sufficiently effective to inhibit the antimicrobial activity of the oral gel. For *S. aureus,* even the use of the specific neutralizer did not enable the neutralization validation required. The standard ISO 11930 states that in this case, the preservative system is considered as sufficiently protective for the product against the pathogenic agent in question.

In accordance with the ISO 11930 standard, KLIRICH is deemed safe and properly preserved according to the criterion A (SCIENTIS report 080-1REA14).

Logarithmic reduction rate (R_X = lg N_O - lg N_X) required[a]								
Microorganisms	Bacteria			C. albicans			A. brasilienis	
Sampling time	T7	T14	T28	T7	T14	T28	T14	T28
Criteria A	≥3	≥3 and PA[b]	≥3 and PA	≥1	≥1 and PA	≥1 and PA	≥0[c]	≥1
Criteria B	Not performed	23	23 and PA	Not performed	≥1	21 and PA	20	20 and PA

a In this test, a range of deviation of 0.5 log is acceptable (See 5.7).
b PA: no increase in the number of microorganisms relative to the previous contact time.
c R_X = 0 when $lgN_O = lgN_X$ (no increase relative to the initial number).

The challenge test performed on the definitive formulation of KLIRICH justified an antimicrobial protection that complies with the requirements of the ISO 11930 standard, the preserving of KLIRICH is therefore ensured.

EFFICACY TESTS

The study of the antimicrobial efficacy of KILIRICH was carried out without xanthan gum or without xantham gum and sodium hyaluronate (with no antimicrobial activity) because ~~this~~ these raw materials cause~~s~~ a gelling making the technique more complex.

In order to take into account the short lifetime of the product in the oral cavity after application, the contact times arbitrarily set during the tests were 30, 45 and/or 60 seconds.

Candida albicans

Carrier tests were conducted according to the methodology of the EN 14562 standard.

Candida albicans	Testes concentration	Logarithmic reduction
Contact time : 60 seconds	100%	3,02

The results (SCIENTIS report 019-1REA15) showed a logarithmic reduction greater than 3, which corresponds to a 99.90% reduction of the load of microorganisms relative to the initial inoculum.

Under the test conditions, the oral gel therefore has a yeasticidal activity against *C. albicans,* which is responsible for oral candida.

Streptococcus mutans

Suspension tests were performed according to the methodology of the standard EN 13727[(3)] in membrane filtration, 2% of NaDBS was added to the rinsing liquid to improve the neutralization of the product.

It was checked that this substance was not toxic to the tested microorganism.

After the defined contact time, the product is filtered on nitrocellulose membranes with a 0.45 µm porosity. Hence the tested product flows through the membrane, which makes it possible to stop its contact with the potentially surviving microbial load which will remain on the surface of the membrane to be counted.

A rinsing liquid is necessary in order to wash the membranes before placing them on agars. The first test is in fact a screening for which only the neutralization and toxicity controls were performed. The results showed an absence of neutralization of the oral health gel.

Streptococcus mutans	Tested concentration	Logarithmic reduction
Time contact : 60 seconds	100%	> 3,06
Time contact : 30 seconds	100%	> 3,05

The results (Reports SCIENTIS 050-1REA15 and 051-1REA15) lead to the conclusion that the gel has a bactericidal activity from 30 seconds (reduction of more than 99.90% of the initial microbial load) against the strain *S. mutans,* responsible for the development of dental caries.

Prevotella melaninogenica

Membrane filtration tests according to the standard EN 13727[(3)] were performed.

Prevotella melaninogenica	Tested concentration	Logarithmic concentration
Contact time : 60 seconds	100%	> 4.25
Contact time : 30 seconds	100%	> 4.22

The results (Report MIDAC RE 15251-3) lead to the conclusion that the gel has a bactericidal activity from 30 seconds (reduction of more than 99.99% of the initial microbial load) against the strain *P. Melaninogenica.*

Porphyromonas gingivalis

Membrane filtration tests according to the standard EN 13727[(3)] were performed 3 times on the same batch of a KLIRICH.

	Tested concentration	Logarithmic concentration
Contact time : 30 seconds	100%	> 5.50
	100%	> 5.32
	100%	> 5.36

The results (reports SCIENTIS 165-1REA14, 165-2REA and 165-3REA14) lead to the conclusion that the gel has a bactericidal activity in 30 seconds (reduction of more than 99.999% of the initial microbial load) against the strain *P. Melaninogenica.*

Streptococcus sanguinis

Membrane filtration tests according to the standard EN 13727[(3)] were performed 3 times on the same batch of a KLIRICH without sodium hyaluronate.

Streptococcus sanguinis	Tested concentration	Logarithmic concentration
Contact time : 30 seconds	100%	> 5.24
	100%	> 5.19
	100%	> 5.04

The results (Report SCIENTS 166-1REA14, 166-2REA14 and 166-3RE14) lead to the conclusion that the gel has a bactericidal activity in 30 seconds (reduction of more than 99.999% of the initial microbial load) against the strain *S. sanguinis.*

Actinomyces naeslundii

Membrane filtration tests according to the standard EN 13727[(3)] were performed.

Actinomyces naeslundii	Tested concentration	Logarithmic concentration
Contact time : 60 seconds	100%	> 5,37
Contact time : 30 seconds	100%	> 5,37

The results (Report MIDAC RE 16191-1) lead to the conclusion that the gel has a bactericidal activity from 30 seconds (reduction of more than 99.999% of the initial microbial load) against the strain *Actinomyces naeslundii.*

Actinomyces israelii

Membrane filtration tests according to the standard EN 13727[(3)] were performed.

Actinomyces israelii	Tested concentration	Logarithmic concentration
Contact time : 60 seconds	100%	> 5,07
Contact time : 30 seconds	100%	> 4,71

The results (Report MIDAC RE 16191-1) lead to the conclusion that the gel has a bactericidal activity from 30 seconds (reduction of more than 99.998% of the initial microbial load) against the strain *Actinomyces israelii.*

Aggregatibacter actinomycetmcomitans

Membrane filtration tests according to the standard EN 13727[(3)] were performed.

Aggregatibacter actinomycetmcomitans	Tested concentration	Logarithmic concentration
Contact time : 60 seconds	100%	> 5,38
Contact time : 30 seconds	100%	> 5,38

The results (Report MIDAC RE 16204-1) lead to the conclusion that the gel has a bactericidal activity from 30 seconds (reduction of more than 99.999% of the initial microbial load) against the strain *Aggregatibacter actinomycetmcomitans*

Herpes simplex type 1

The search for a potential virucidal activity of KLIRICH was targeted on a strain of interest, the strain *Herpes simplex* type 1, corresponding to the most common herpes: the oral herpes.
No medical treatment eliminates the virus *Herpes simplex* type 1 from the body in a definitive manner. Nevertheless, the aim here was to prove that KLIRICH is a solution for reducing the symptoms and for relieving the relative pain.
Given the demonstrated bactericidal activity and the directions for use of KLIRICH, three contact times were tested: 30, 45, and 60 seconds.

Herpes simplex virus 1	Tested concentration	Logarithmic concentration
Contact time : 45 seconds	100%	> 4.25
	100%	> 4.125

The tests finally lead to the conclusion that KLIRICH has a specific virucidal activity from 45 seconds against the strain *Herpes simplex* type 1 corresponding to a reduction of more than 99.99% of the initial microbial load (Report APEX BIOLUTIONS 114V09-2014-01).

DISCUSSION

Several difficulties were encountered during the identification of this antimicrobial efficacy.

First of all, the culturing of the above-mentioned microorganisms of the oral sphere, which are not usually tested, in the presence of a reasoned quantity of interfering substance (blood), has not been easy and it has been necessary to develop different and specific culture conditions, particularly for the strains *P. melaninogenica, P. gingivalis, and S. sanguinis.*

Secondly, the neutralization of the antimicrobial activity of the tested products at the end of the claimed contact time had to be developed. Indeed, the complexity of the formulation required the formulation of adapted neutralizers in order to validate the results observed during the tests (without neutralization, the antimicrobial efficacy in a given contact time is not justified).

Thirdly, for reasons of technical feasibility *in vitro*, KLIRICH was tested non-viscosed, therefore without xanthan gum. On the other hands, for the strains *P. gingivalis* and *S. sanguinis,* KLIRICH was tested with neither xantham gum nor sodium hyaluronate. The hypothesis put forward is related to the steric hindrance of the molecule which interfered with the neutralization of the product.

CONCLUSION / SYNTHESIS OF THE RESULTS

Under the conditions of the studies carried out, KLIRICH destroys 99.9% to 99.999% of the microorganisms tested.

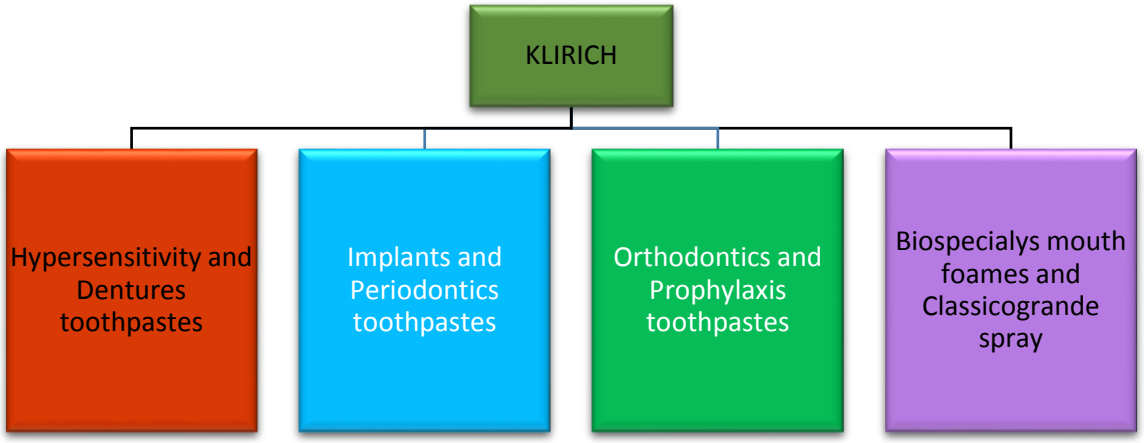
Strain	**Contact time**	**Tested concentration**	**Logarithmic concentration**
Candida albicans	60 seconds	100%	3,02
Streptococcus mutans	30 seconds	100%	> 3,05
	60 seconds	100%	> 3,06
Prevotella melaninogenica	30 seconds	100%	> 4.22
	60 seconds	100%	> 4.25
Porphyromonas gingivalis	30 seconds	100%	> 5.50
		100%	> 5.32
		100%	> 5.36
Streptococcus sanguinis	30 seconds	100%	> 5.24
		100%	> 5.19
		100%	> 5.04
Actinomyces naeslundii	60 seconds	100%	> 5,37
	30 seconds	100%	> 5,37
Actinomyces israelii	60 seconds	100%	> 5,07
	30 seconds	100%	> 4,71
Aggregatibacter actinomycetmcomitans	60 seconds	100%	> 5,38
	30 seconds	100%	> 5,38
Herpes simplex virus 1	45 seconds	100%	> 4.25
		100%	> 4.125

COLATERAL APPLICATION OF KLIRICH

Owing to the properties previously described, KLIRICH was introduced in a range of cosmetic oral health products (toothpastes and mouthwashes).

It is the treatment product of the SPECIALYS range. Different segments were defined:

- ✓ Implants: Avoiding peri-implantitis;
- ✓ Orthodontics: Washing the inside of bands;
- ✓ Dentures: Controlling halitosis and opportunistic oral microorganisms;
- ✓ Prophylaxis: Exfoliating, smoothing and protecting the enamel
- ✓ Periodontics: Preventing and acting on bleeding.
- ✓ Hypersensitivity: Preventing and acting on thermal sensitivity



GENERAL CONCLUSION

Nine pathogenic microorganisms in relation with the oral cavity were tested in vitro: *C. albicans*, *S. mutans*, *P. melaninogenica*, *P. gingivalis, S. sanguinis, Actinomyces naeslundii, Actinomyces israelii, Aggregatibacter actinomycetmcomitans and Herpes Virus* type 1.
Under the conditions of the studies carried out, KLIRICH destroys 99.9% to 99.999% of the microorganisms tested.

A clinical study was carried out in parallel, demonstrating the activity of the product on gingivitis.

KLIRICH, a patented treatment product (publication EP 2954902 A1), was also incorporated in the whole range of dental care products intended to be prescribed by dental art professionals.

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ABBREVIATIONS

°C: Celsius Degree
AFNOR : Association Française de NORmalisation (French Association for Standardization)
ATCC: American Type Culture Collection
CIP: Collection de l'Institut Pasteur (Collection of the Pasteur Institute)
CPC: Cetylpiridinium chloride
CFU : Colony-forming unit
OD: Optical density
MD: Medical device
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen (German collection of microorganisms and cell cultures)
IS: Interfering substance
ISO: International Organization for Standardization
Log: decimal logarithm
mL: Milliliter
MEA: Malt Extract Agar
NaDBS: Sodium dodecylbenzenesulfonate
NB: number
PDA: Potato Dextrose Agar
R0, R1 and R2: Planting out 0, Planting out 1 and Planting out 2.
SUBCULTURE: culture obtained after an isolation or a preceding culture.
T0: Time zero
TS: Tryptone Salt
TSA: Trypticase Soy Agar
TSB: Tryptic Soy Broth