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Campus de Araçatuba

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Influência de concentrações subinibitórias de agentes antimicrobianos sobre a fisiologia e genética de *S. mutans*

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Influência de concentrações subinibitórias de agentes antimicrobianos sobre a fisiologia e genética de *S. mutans*

Tese de doutorado apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP como parte dos requisitos para obtenção do título de Doutor em Ciência Odontológica, na Área de Concentração Biomateriais.

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“Aprendi que um homem só tem o direito de olhar um outro de cima para baixo para ajudá-lo a levantar-se.”

Gabriel García Marquez

Gazola Filho, J. Influência de concentrações subinibitórias de agentes antimicrobianos sobre a fisiologia e genética de *S. mutans*. 2016. 71p. Tese (Doutorado em Ciência Odontológica) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2016.

RESUMO

Streptococcus mutans é conhecido por sua capacidade de aderir, colonizar e formar biofilme nas superfícies dentárias. Para sobreviver na cavidade bucal, *S. mutans* coordena a expressão de vários genes em resposta aos fatores ambientais, como na presença de agentes antimicrobianos. Os objetivos deste estudo foram: 1) avaliar a influência de concentrações subinibitórias (sub-MIC) de fluoreto de sódio (NaF) e digluconato de clorexidina (Chx) sobre o crescimento planctônico; 2) avaliar a influência desses antimicrobianos em concentrações sub-MIC na formação de biofilme das cepas de *S. mutans*; 3) avaliar a expressão dos genes *covR* e *vicR* de cepas de *S. mutans* expostas aos sub-MIC de Chx e NaF. Cepas de *S. mutans* padrão (ATCC 25175 e UA159) e isolada de criança com cárie precoce da infância (3FV2) foram reativadas em BHI caldo e expostas a Chx e NaF para determinação da MIC e MBC. A partir desses valores, curvas de crescimento foram obtidas para as cepas de *S. mutans* expostas a concentrações sub-MIC de Chx (0,25 x MIC a 0,75 x MIC) e NaF (0,125 x MIC a 0,75 x MIC). Ensaios de biofilme foram realizados para as cepas de *S. mutans* expostas ou não aos sub-MIC dos agentes antimicrobianos (0,25 x MIC; 0,5 x MIC e 0,75 x MIC). A expressão dos genes *covR* e *vicR* foram avaliadas por PCR quantitativo para as cepas de *S. mutans* em condições planctônicas expostas a 0,125/0,25/0,5 x MIC de NaF e Chx. Os dados obtidos foram submetidos à análise estatística considerando $p \leq 0,05$. Os resultados mostraram que sub-MIC de Chx e de NaF afetaram o crescimento planctônico das cepas de *S. mutans*, de maneira dose-dependente. Sub-MIC de Chx afetou somente a formação de biofilme de 3VF2 e valores de NaF acima de 0,5 x MIC causaram redução do crescimento do biofilme para todas as cepas testadas, de maneira dose-dependente, com exceção da cepa ATCC 25175, que foi afetada igualmente por todas as concentrações avaliadas. Concentrações sub-MIC gradativas de Chx induziram a expressão crescente do gene *vicR* das cepas avaliadas. Quando NaF e Chx foram associados, o gene *covR* teve sua expressão reduzida quando expostas a 0,5 x MIC. Conclui-se que concentrações sub-MICs de NaF acima de 0,5 x MIC causaram redução do crescimento planctônico e em biofilme das cepas de *S. mutans*. Concentrações sub-MIC de Chx induzem a expressão de *vicR* podendo favorecer a formação de biofilme pelas cepas de *S. mutans.*

Palavra-Chaves: *Streptococcus mutans*, cárie dentária, teste de sensibilidade microbiana, fluoreto de sódio, clorexidina.

Gazola Filho, J. Influence of subinhibitory concentrations of antimicrobial agents on the physiology and genetics of *S. mutans*. 2016. 71p. Tese (Doutorado em Ciência Odontológica) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2016.

ABSTRACT

Streptococcus mutans is known for its ability to adhere, colonize and form biofilm on dental surfaces. To survive in the oral cavity, *S. mutans* coordinates the expression of several genes in response to environmental factors, such as in the presence of antimicrobial agents. The objectives of this study were: 1); to evaluate the influence of subinhibitory concentrations (sub-MIC) of chlorhexidine (Chx) and sodium fluoride (NaF) on planktonic growth 2) to evaluate the influence of sub-MIC of these agents on biofilm formation of *S. mutans* strains; 3) to evaluate the expression of the *covR* and *vicR* genes of *S. mutans* strains exposed to sub-MIC of Chx and NaF. Standard strains of *S. mutans* (ATCC 25175 or UA159) and clinical strain isolated from child with caries (3FV2) were reactivated in BHI broth and exposed to Chx and NaF for determination of MIC and MBC. From these values, growth curves were obtained for *S. mutans* strains exposed to sub-MIC concentrations of Chx (0.25 x MIC to 0.75 x MIC) and NaF (0.125 x MIC to 0.75 x MIC). Biofilm assays were performed for *S. mutans* strains exposed or not to the sub-MIC of antimicrobial agents (0.25, 0.5 and 0.75 x MIC). Expression of *covR* and *vicR* genes were evaluated by quantitative PCR for strains of *S. mutans* under plankton conditions exposed to 0.125 / 0.25 / 0.5 x MIC of NaF and Chx. The data were submitted to statistical analysis considering $p \leq 0.05$. The results showed that Chx and NaF sub-MIC affected planktonic growth of *S. mutans* strains in a dose-dependent manner. Sub-MIC of Chx only affect the biofilm formation for ATCC 25175, and, NaF values above 0.5 x MIC caused reduction of the biofilm growth for all tested strains, in a dose-dependent manner, except for ATCC 25175, which was also affected by all concentrations evaluated. Gradual sub-MIC concentrations of Chx induced increasing expression of the *vicR* gene of the strains evaluated. When NaF and Chx were related, the *covR* gene had its expression reduced when exposed to 0.5xMIC. It is concluded that sub-MICs concentrations of NaF above 0.5 x MIC caused reduction of planktonic and biofilm growth of *S. mutans* strains. Sub-MICs concentrations of Chx induce *vicR* expression and may favor biofilm formation by *S. mutans* strains.

Keywords: *Streptococcus mutans*, dental caries, microbial sensitivity test, fluoride, chlorhexidine.

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LISTA DE ABREVIATURAS

µg	Micrograma
µl	Microlitro
Abs	Absorbância
ANOVA	Análise de Variância
ATCC	*American Type Culture Collection*
BHI	*Brain Heart Infusion*
BHIA	*Brain Heart Infusion Agar*
C	*Celsius*
cDNA	*Complementary Deoxyribonucleic Acid*
Chx	Clorexidina
CovR	*Control of Virulence*
CovRS	*Control of Virulence*
covS	*Control of Virulence*
DEPC	*Diethylpyrocarbonate*
DNase	*Deoxyribonuclease*
DO	Densidade óptica
EDTA	*Ethylenediamine tetraacetic acid*
ELISA	*Enzyme Linked ImmunonoSorbent Assay*
EPS	Exopolissacarídeos
FIOCRUZ	Fundação Oswaldo Cruz
ftf	Frutosiltransfare
GbpB	*Glucan-binding protein B*
Gbps	*Glucan-binding proteins*
GtfB	Glicosiltransferase B
GtfC	Glicosiltransferase C
GtfD	Glicosiltransferase D
GTFs	Glicosiltransferases
h	Horas
H_2O	Água
HCl	Ácido Clorídrico
IgA	Imunoglobulina A

MAS	*Marker Aided Selection*
MBC	Mínima concentração bactericida
MIC	Mínima concentração inibitória
Mili-Q	Água deionizada
Min.	Minutos
ml	Mililitro
MOPS	*3- Propanesulfonic Acid*
MSB	Mitis Salivarius Agar com 2U/L de bacitracina
NaCl	Cloreto de Sódio
NaF	Fluoreto de Sódio
Nm	Nanômetro
pb	Pares de base
Ph	Potencial Hidrogeniônico
qPCR	Proteína C Reativa Quantitativa
Qsp	Quantidade Suficiente Para
RNAm	*Ribonucleic Acid Messenger*
RT	Reverse transcription
S	Segundos
S. mutans	*Streptococcus mutans*
sub-MICs	Concentrações subinibitórias mínimas
TCS	*Two component systems*
TE	*Tris-EDTA*
U	Unidade
ul	Microlitro
UFC	Unidades Formadoras de Colônias
UV	Ultra Violeta
VicR	*Virulence control Regulator*
VicX	*Virulence control*
vicK	*Virulence control Kinase*
VicRK	*Virulence control Regulator Kinase*
Vic	*Virulence control*

SUMÁRIO

ARTIGO*

Influência de concentrações subinibitórias de agentes antimicrobianos no crescimento, formação de biofilme e expressão de covR/vicR de *S. mutans*

Influence of subinhibitory concentrations of antimicrobial agents on the growth, biofilm formation and *cov*R/*vic*R expression of *S. mutans*

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RESUMO

Objetivos: avaliar a influência de concentrações subinibitórias (sub-MIC) de fluoreto de sódio (NaF) e digluconato de clorexidina (Chx) sobre o crescimento planctônico, na formação de biofilme e na expressão dos genes *covR* e *vicR* de cepas de *S. mutans* expostas aos sub-MIC de Chx e NaF.

Métodos: Cepas de *S. mutans* (ATCC 25175, UA159 e 3VF2) foram reativadas em BHI caldo e expostas a Chx e NaF para determinação das Concentração Inibitória Mínima (MIC) e Concentração Bactericida Mínima (MBC). A partir desses valores, curvas de crescimento foram obtidas para estas cepas expostas a concentrações sub-MIC de Chx (0,25 x MIC a 0,75 x MIC) e NaF (0,125 x MIC a 0,75 x MIC). Ensaios de biofilme foram realizados para as cepas expostas ou não aos sub-MIC dos agentes antimicrobianos (0,25 x MIC; 0,5 x MIC e 0,75 x MIC). A expressão dos genes *covR* e *vicR* foi avaliada por PCR quantitativo para as cepas expostas a 0,125/0,25/0,5 x MIC de NaF e Chx. Os dados obtidos foram analisados estatisticamente, considerando $p<0,05$.

Resultados: Sub-MIC de Chx e de NaF afetaram o crescimento planctônico das cepas, de maneira dose-dependente. Sub-MIC de Chx afetou somente a formação de biofilme de 3VF2 e valores de NaF acima de 0,5 x MIC causaram redução do crescimento do biofilme para todas as cepas testadas, com exceção da cepa ATCC 25175, que foi afetada igualmente por todas as concentrações avaliadas. Concentrações sub-MIC gradativas de Chx induziram a expressão crescente do gene *vicR* das cepas avaliadas. Quando NaF e Chx foram associados, o gene *covR* teve sua expressão reduzida quando estas foram expostas a 0,5 x MIC.

Conclusão: Conclui-se que concentrações sub-MIC de NaF e Chx causaram redução do crescimento planctônico para todas as cepas e em biofilme das cepas de *S. mutans*. Sub-MIC de Chx induziram a expressão de *vicR* podendo favorecer a formação de biofilme por *S. mutans.*

INTRODUÇÃO

As bactérias são frequentemente expostas a vários tipos de estresse que desafiam sua capacidade de sobrevivência. A habilidade de adaptar sua fisiologia celular e comportamento frente a esses estímulos determina a persistência de algumas espécies e a eliminação de outras [1, 2]. Na cavidade bucal, os microrganismos são também submetidos a condições de estresse, como o calor, alterações de pH, estresse osmótico ou oxidativo e alternativamente, à substâncias antimicrobianas [3, 4]. Estas substâncias podem estar contidas nos produtos de higiene bucal, como os fluoretos, clorexidina, entre outros, ou ainda serem derivadas de células imunológicas do próprio hospedeiro, que agem como antimicrobianos naturais. Ambas as formas de antimicrobianos, sintéticas ou naturais, atuam diretamente no controle da proliferação dos microrganismos [3, 4].

Entre os agentes anticárie, os que apresentam maior aplicação em clínica odontológica são a Chx e o NaF. A clorexidina é uma bisbiguanida catiônica com ampla atividade antibacteriana, que reage com os grupos carregados negativamente na superfície celular e causa uma perda irreversível dos constituintes citoplasmáticos, danos na membrana e inibição enzimática. A clorexidina é um dos agentes antimicrobianos mais seguros e efetivos contra estreptococos orais [5, 6], principalmente do grupo *mutans*. Além disso, também é capaz de atuar contra outras espécies Gram positivas, Gram negativas, fungos e leveduras, aeróbias facultativas e anaeróbias [7]. O flúor é o elemento mais eletronegativo dos halogênios, ele reage facilmente com outros elementos químicos. O fluoreto de sódio é o mais importante agente anticárie disponível, pois retarda desmineralização dentária, atua na reimineralização do dente e possui ação antibacteriana [8]. A ação antimicrobiana do NaF está relacionada com inibição da utilização de carboidratos pelos microrganismos orais pelo bloqueio de enzimas envolvidas na via glicolítica bacteriana [8, 9].

Um mecanismo de sobrevivência aos estresses é a formação dos biofilmes, ou seja, estruturas tridimensionais complexas compostas pela agregação de populações microbianas embebidas uma matriz hidratada de exopolissacarídeos (EPS) [10, 11] que levam ao desenvolvimento de características únicas como a redução na susceptibilidade aos antimicrobianos e biocidas comparados aos organismos planctônicos [12-14]. Um pré-requisito ideal para um tratamento antimicrobiano de sucesso é que todas as bactérias do biofilme sejam expostas a uma concentração adequada por tempo suficiente. Os mecanismos conhecidos de resistência do biofilme aos antimicrobianos incluem as barreiras físicas ou químicas para a penetração antimicrobiana no biofilme, o crescimento

lento do biofilme devido à limitação de nutrientes, a ativação da resposta ao estresse e o surgimento de um fenótipo bacteriano específico no biofilme [15-17].

Streptococcus mutans é considerado o principal agente etiológico da cárie dentária devido a sua capacidade de se aderir e acumular no biofilme dental, de formar ácidos que promovem a desmineralização dentária, além de tolerar os ambientes com baixo pH, mantendo seu metabolismo em condições adversas. Para se estabelecer na cavidade bucal, *S. mutans* sintetizam polissacarídeos a partir da sacarose proveniente da dieta. Para isso, são produzidas as enzimas, GtfB e GtfC (glicosiltransferase), que atuam na síntese de glicanos insolúveis em água e GtfD na síntese de glicanos solúveis em água. Os primeiros glicanos estão diretamente associados com a formação da matriz de EPS do biofilme dental. *S. mutans* também produz proteínas de superfície que apresentam afinidade aos glicanos, as Gbps (de glucan binding proteins), que medeiam à interação bacteriana com o glicano extracelular e contribuem para o crescimento do biofilme [18, 19]

Alguns estudos têm demonstrado que agentes antimicrobianos em concentrações subinibitórias mínimas (sub-MIC) podem efetivamente inibir a formação de biofilme [20, 21], já outros mostraram que esses agentes nessas concentrações podem induzir a formação de biofilme [22, 23]. Entretanto, somente um desses estudos, o realizado por Dong et al. [23], avaliou a influência dos sub-MIC de agentes anti-cárie (clorexidina, fluoreto de sódio, polifenóis de chás) sobre *S. mutans*, em estado planctônico ou em biofilme. Os agentes regularam positivamente a expressão de genes relacionados com a formação de biofilme e em microscopia eletrônica de varredura foi verificado que *S. mutans* exibiram um biofilme denso com uma extensa matriz extracelular após a exposição aos agentes, demonstrando que houve estímulo para o crescimento bacteriano quando utilizadas concentrações baixas de antimicrobianos.

S. mutans apresenta uma considerável capacidade de coordenar a expressão de vários genes em resposta aos fatores ambientais. Para se adaptarem aos estresses durante a colonização do hospedeiro e formação de biofilme, as bactérias apresentam sistemas de regulação gênica denominados de sistemas de dois componentes (TCS – two component systems) [24]. O genoma de *S. mutans* (cepa UA159) apresenta 14 TCS completos, incluindo o TCS denominado VicRK (Vic - virulence control) e o CovR (control of virulence) [25]. Esses dois sistemas, VicRK e CovRS, regulam diretamente um painel de genes relacionados com a síntese e a interação com os polissacarídeos extracelulares [19, 26, 27]. A maioria dos genes são positivamente regulados por VicRK e/ou reprimido por CovR (*gtfB/C/D, gbpC*) [26, 28]. Stipp et al. [29] avaliaram diversas funções dos sistemas CovR e VicRK e verificaram que esses sistemas regulam um grande número de genes

envolvidos na biogênese da parede celular que são especificamente ativados durante o crescimento na fase de formação do biofilme. Nesse estudo, cepas *knockout* desses TCS apresentaram reduzida formação de microcolônias e fragilidade do biofilme. Duque et al. [28] exploraram a estratégia do RNA antisense para analisar as funções biológicas de *gbpB* e observaram que a proteína GbpB e participa da formação do biofilme sacarose-dependente e de outras propriedades celulares, como controle da hidrofobicidade celular, resistência ao estresse oxidativo e osmótico, à autólise e sensibilidade à antibióticos. Também verificaram que *gbpB* é regulada pelo sistema VicRK . Ainda não existem estudos mostrando se esses sistemas VicRK e CovRS e consequentemente os genes que eles regulam são influenciados positiva ou negativamente pela presença de antimicrobianos provenientes de produtos de higiene oral.

Este estudo objetivou avaliar a influência de concentrações subinibitórias dos agentes antimicrobianos sobre crescimento planctônico (em curvas de crescimento), a formação de biofilme das cepas de *S. mutans* e a expressão dos genes *covR* e *vicR* de cepas de *S. mutans* expostas aos sub-MIC dos agentes antimicrobianos.

MATERIAL E MÉTODOS

Cepas Bacterianas e Condições de Crescimento

Foram utilizadas para este estudo as seguintes cepas de *S. mutans:* uma cepa padrão (ATCC 25175), cedida pela Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro - RJ, a cepa sequenciada (UA159) [25] e uma cepa clínica (3VF2) isolada de criança com cárie severa da infância, ambas gentilmente cedidas pela Profa. Dra. Renata de Oliveira Mattos-Graner. A cepa clínica 3VF2 foi diferenciada geneticamente e fisiologicamente em estudo prévio [30]. As cepas de *S. mutans* foram reativadas de uma suspensão estocada a -70^{o} C e crescidas em placas de MSB (Mitis Salivarius Agar com 2U/L de bacitracina) por 24h em estufa com 5% CO_2 e 95% de ar. Uma única colônia foi inoculada em 5mL de BHI (Brain Heart Infusion, Difco, Detroit, MI) caldo e incubadas por 24h, antes do ensaios laboratoriais.

Preparo dos Agentes Antimicrobianos

Soluções-estoque dos agentes antimicrobianos foram preparadas nas seguintes concentrações: 20mg/mL de solução de fluoreto de sódio – NaF e 1mg/ml de digluconato de clorexidina (Chx). Todos os produtos citados foram comprados da Sigma-Aldrich Chemical Col, St. Louis, MO, diluídos em água destilada estéril e filtrados usando filtro descartável com poro de 0,22um (Corning Cambridge, MA) e estocadas a 4^{o} C [23].

Ensaios de MIC (mínima concentração inibitória) e MBC (mínima concentração bactericida).

Os ensaios de MIC foram baseados nos critérios descritos pelo National Committee for Clinical Laboratory Standards para bactérias [31], em triplicata. Culturas das espécies bacterianas foram repicadas em meio BHI (Difco) por 24h a 37^{o} C. Quando a DO $_{550nm}$ = 0.5 (aproximadamente 10^8 células) for atingida, a suspensão bacteriana foi diluída em caldo BHI fresco (1:1000). Os ensaios de MIC foram obtidos pelo método de microdiluição. Resumidamente, alíquotas de 50ul de células bacterianas ($2x10^5$ a $7x10^5$ UFC – unidades formadoras de colônias, dependendo da cepa utilizada) foram incubadas em 50ul da diluição seriada dos agentes antimicrobianos dissolvidos. Após incubação por 24h a 37^{o} C, as placas de microtitulação foram analisadas a 550nm em um leitor de microplacas (Eon Spectrophotometer,Biotek). Cada ensaio foi realizado em triplicata. Para controle, suspensões bacterianas foram incubadas em água Mili-Q estéril. MIC foi definida como a menor concentração do agente antimicrobiano que não houve crescimento detectável

(leitura menor que 0,05). Os meios contendo essa concentração de MIC e duas concentrações posteriores foram diluídas seriadamente e plaqueadas em meio BHIA e incubadas a 37^{o} C por 48h para obtenção da MBC. As UFC foram contadas com auxílio de estereomicroscópio binocular.

Curvas de Crescimento Planctônico

Culturas das cepas de *S. mutans* descritas anteriormente (DO $_{550nm}$ = 0,5 ou aproximadamente 10^8 células/ml) foram inoculadas em tubos contendo BHI em uma diluição de 1:100 da bactéria. Em seguida, uma solução de cada antimicrobiano foi adicionada aos tubos, mantendo a concentração de 0,125 x MIC; 0,25 x MIC; 0,5 x MIC e 0,75 x MIC de NaF e de 0,25 x MIC; 0,5 x MIC e 0,75 x MIC de Chx. Os tubos (em duplicata) foram incubados a 37^{o}C por 48h. As leituras das densidades ópticas foram obtidas nos tempos 2,4,6,8, 10, 24 e 48h. Tubos controle (sem agentes antimicrobianos) foram incluídos no estudo. Os experimentos foram realizados em triplicata [23].

Formação de biofilme in vitro

Culturas das três cepas de *S. mutans* (DO $_{550nm}$=0,5) foram diluídas em meio BHI com 0,2% de sacarose (obtenção de 10^7 células/ml) e 100 µl de cada cultura foram inoculados individualmente nos poços forrados com a película adquirida e mantida em estufa 5% CO_2 e 95% de ar por 3 horas a 37°C. Em seguida, o meio nas placas foram substituído por 200 µl de meio BHI fresco com 0,2% de sacarose contendo ou não agentes antimicrobianos nas MICs e sub-MICs (0,25 x MIC; 0,50 x MIC; 0,75 x MIC). As placas foram incubadas por 24h, e o meio de cultura trocado e mantidas até 48 horas em condições anaeróbias. Após esse período, as placas foram lavadas 3 vezes com 200 µl de PBS por poço e analisadas para quantificação da biomassa do biofilme. Para as análises da biomassa de biofilme, as amostras foram crescidas em placas de fundo redondo [30].

Quantificação da Biomassa do Biofilme Formado

Após breve secagem das placas previamente lavadas com PBS, foram adicionados 150 µl por poço de solução aquosa de cristal violeta a 1% e as placas incubadas em temperatura ambiente durante 30 min. A seguir, a solução de cristal violeta foi removida e as placas lavadas novamente por 3 vezes. As placas foram mantidas invertidas sobre papel toalha durante 2 h em temperatura ambiente, para secagem. A seguir, o corante de cristal violeta foi solubilizado dos biofilmes corados através da incubação com 200 µl de etanol 95% por poço durante 30 min. Então, volumes de 100 µl das soluções do corante

em etanol foram transferidos para poços de uma nova placa, as quais foram submetidas à leitura das absorbâncias a 575nm, em leitor de ELISA (Eon Spectrophotometer, Biotek). A absorbância das suspensões celulares para cada cultura foram medidas como um controle para o crescimento planctônico [30].

Análise da Expressão de Genes Relacionados à Formação de Biofilme

Extração e Purificação de RNA

Para a extração do RNA total das cepas de *S. mutans* (ATCC, UA159 e 3VF2) foram utilizadas culturas até metade da fase logarítmica de crescimento (A_{550nm} = 0,3) em 10% CO_2 na presença ou não dos antimicrobianos (NaF e Chx) nas suas respectivas MIC e sub-MIC (0,25 x MIC; 0,5 x MIC; 0,75 x MIC). Para isto, as cepas foram reativadas em placas de MSA e posteriormente inoculadas em meio BHI acrescidos de flúor e clorexidina, e incubadas por 18 h para a obtenção da cultura *overnight* de cada cepa. As culturas foram normalizadas (A_{550nm} =0,035) em 30 ml de caldo BHI acrescidos de flúor e clorexidina nas diferentes concentrações citadas acima e incubadas em 10% CO_2 até A_{550nm}= 0.3. Os tubos contendo 5 mL das culturas foram centrifugadas (Centrífuga Eppendorf 5810R) 16.000 xg, 5 min. 4°C e os *pellets* ressuspendidos em 1 ml de solução salina (NaCl 0,9%) e as células foram transferidas para tubos de microcentrífuga com rosca de capacidade de 2 ml (*Screw Tube, Axygen*, EUA), sendo então centrifugadas (16.000 xg, 1 min, 4°C). O sobrenadante das culturas foi descartado, e os *pellets* congelados em ultra-freezer -70°C para posterior purificação do RNA [32].

Posteriormente, as células foram lisadas na presença de aproximadamente de 0,16 g de esferas de zircônia (0,1 mm de diâmetro) (Biospec, EUA) adicionadas de 200 µl de TE (Tris- HCl 10 mM pH 8,0; EDTA 1 mM pH 8,0) em aparelho Mini-bead beater (Biospec, EUA) em força máxima (2 ciclos de 30 s com 30 s de descanso em gelo). A purificação do RNA total foi feita utilizando o Kit *RNeasy Mini Kit* (Qiagen, Alemanha) seguindo o protocolo do fabricante. O RNA purificado foi tratado com 10 U de Turbo *DNase* (Invitrogen), para a eliminação total de DNA genômico. A leitura para a determinação da concentração e pureza das amostras foi feita em aparelho Nanodrop (Thermo Scientific, EUA). A faixa limite para a consideração das amostras puras foi a razão entre as leituras A_{260nm}/A_{280nm} (> 1,8). A integridade das amostras de RNA foi avaliada em géis de agarose a 1,2% (com 1,8% de formaldeído), contendo 0,15 µg/ml de brometo de etídio, após separação eletroforética em tampão de corrida contendo 20 mM MOPS, 5 mM de acetato de Sódio e 1 mM de EDTA. Imagens dos géis foram obtidas sob luz UV e a presença de

duas bandas definidas, correspondentes aos RNAs ribossômicos (23S e 16S) indicaram a integridade das amostras [32].

Obtenção de cDNA

As reações de transcriptase reversa foram realizadas com as amostras de RNAs total utilizando-se os *primers* (Invitrogen, Brasil) arbitrários Ea1 (5'-TTTTATCCAGC-3'), Ea7 (5'-TCTTTTTTACC-3'), Es1 (5'-GCTGGAAAAA-3'), Es3 (5'-GAAGTGCTGG-3') e Es8 (5'TGCCGATGAA-3'), como descrito em estudos anteriores (32). Para obtenção de um *pool* de cDNA a partir do RNAm total, alíquotas contento quantidades de 5,0 µg de RNA total, foram tratadas com o sistema DNAse I Amp grade (Invitrogen), para eliminar qualquer traço de DNA genômico contaminante, de acordo com as instruções do fabricante. Foram adicionados a cada amostra, 5 µl de enzima DNAse, 3 µl de tampão 10x DNAse e H_2O DEPC qsp 30 µl, sendo as amostras incubadas durante 15 min em temperatura ambiente. A seguir, 4 µl de solução de EDTA a 25 mM foram acrescentados a cada amostra e as mesmas foram incubadas a 65^{o}C durante 10 min, para a inativação da enzima DNAse I. As reações de transcrição reversa foram posteriormente preparadas com o sistema SuperScript III (Invitrogen), seguindo as recomendações do fabricante. Para isto, foram realizadas misturas contendo 2 µg de RNA total livre de DNA, 3 µl da mistura dos *primers* arbitrários (concentração final de 2 µM para cada oligo), 2 µl de 10 mM DNTP mix e H_2O DEPC qsp 26 µl. Cada mistura inicial foi aquecida a 65ºC por 5 min, e resfriada por 1 min em gelo. Um segundo microtubo, contendo 8 µl Buffer 5X, 2 µl de 0.1M DTT, 2 µl de RNase OUT (40 U/µl) e 2 µl de SuperScript III (200 U/µl) por amostra, foi preparado como solução mãe, sendo 14 µl desta solução transferidas para cada tubo resfriado. As reações de RT ocorreram com incubação a 25^{o}C durante 10 min, a 50^{o}C por 3 h e a 85ºC por 5 min em termociclador (Thermoblock T1, Biometra, Alemanha). Os cDNAs obtidos foram estocados a -20^{o}C, para posterior utilização nas reações de PCR quantitativo. Para controle da contaminação por DNA genômico, foram conduzidos em paralelo ensaios para cada amostra de RNA tratado, sob as mesmas condições, substituindo-se 2 µl de SuperScript III (200 U/µl) por 2 µl de H_2O DEPC. Estas amostras foram incluídas, como controle de contaminação, nas reações de PCR quantitativo [32].

Ensaios de PCR Quantitativo (qPCR)

As reações de qPCR foram realizadas a partir do *pool* de cDNA obtido em uma única reação de RT, para cada tempo de cada amostra bacteriana testada, para quantificar

a expressão dos genes estudados, como descrito em estudos anteriores[28]. Para isto, pares de *primers* específicos para cada gene foram delineados com o auxílio do programa *Primer 3,* a partir das sequências do genoma da cepa UA159 disponíveis no banco de dados de *Los Alamos National Laboratory Bucal Pathogens Sequence Database* (http://www.bucalgen.lanl.gov). Cada par de *primer* amplificou sequências entre 163 e 190 pb. Como gene de referência, foi utilizado o 16S, que apresentou número de transcritos constante na fase d

}e crescimento planctônico testada (A_{550nm}=0,3). Os *primers* delineados para esses experimentos foram descritos por Stipp et al. [32].

Para a quantificação dos transcritos de cada gene teste, reações de qPCR contendo cDNA (30 ng), 30 µM de cada par de *primer* e *SYBR-Green PCR Master Mix* (Applied Biosystems) em volume total de 10 µl foram realizadas através do sistema PCR quantitativo StepOne (*Applied Biosystems*). Cada ciclo térmico de amplificação consistiu de desnaturação inicial a 95°C por 10 min, seguida de 40 ciclagens de desnaturação a 94°C por 15 s, anelamento por 15 s e extensão a 72°C por 30 s. Os ensaios de qPCR foram realizados em triplicata, a partir de amostras cDNA obtidas em três experimentos independentes. Como um dos controles negativos da reação, misturas sem cDNA sem transcriptase reversa foram incluídas para assegurar a ausência de DNA contaminante. Para cada corrida, curvas de amplificação de DNA de *S. mutans* em quantidades crescentes (0,03; 0,3; 3; 30 e 300 ng/poço) foram realizadas. Os valores de expressão de cada gene teste foram normalizados pelo gene 16S. Seguindo o modelo matemático proposto por Pfaffl, 2001. Também foram realizadas análises do número de transcritos para a confirmação da expressão dos genes *covR* e *vicR* [32].

Análise Estatística

Comparou-se as cepas de *S. mutans* expostas aos antimicrobianos com aquelas não expostas em relação a formação de biofilme utilizando os testes estatísticos não paramétricos de Kruskal-Wallis e Mann-Whitney. Quando avaliados os níveis de expressão dos genes foi utilizada a análise de variância (ANOVA) seguida do teste de Tukey, comparando-se cepas expostas ou não aos antimicrobianos e as diferentes concentrações dos antimicrobianos.

RESULTADOS

Valores de MIC/MBC

Tabela 1. Valores de MIC/MBC obtidos para os agentes antimicrobianos testados contra as cepas de *S. mutans.*

Cepas de *S. mutans*	NaF(mg/ml)		Chx (µg/ml)	
	MIC	MBC	MIC	MBC
UA159 (padrão)	0,125	0,25	0,03	0,24
ATCC 25175 (padrão)	0,125	2	0,1	0,4
3VF2 (clinica)	0,5	2	0,2	0,9

Na **Tabela 1,** pode-se observar que para NaF, as cepas padrão obtiveram valores de MIC iguais e a cepa clínica 3VF2 maior que as demais, entretanto, para MBC a cepa padrão UA159 foi a mais sensível. Quanto à Chx, os valores de MIC das cepas de *S. mutans* foram diferentes, com destaque para a cepa clínica 3VF2 que foi a menos sensível. O mesmo foi observado para MBC de Chx.

Curvas de Crescimento

As **Figuras 1A a 1C** mostram as curvas de crescimento das cepas de *S. mutans* frente às concentrações subinibitórias de NaF e Chx.

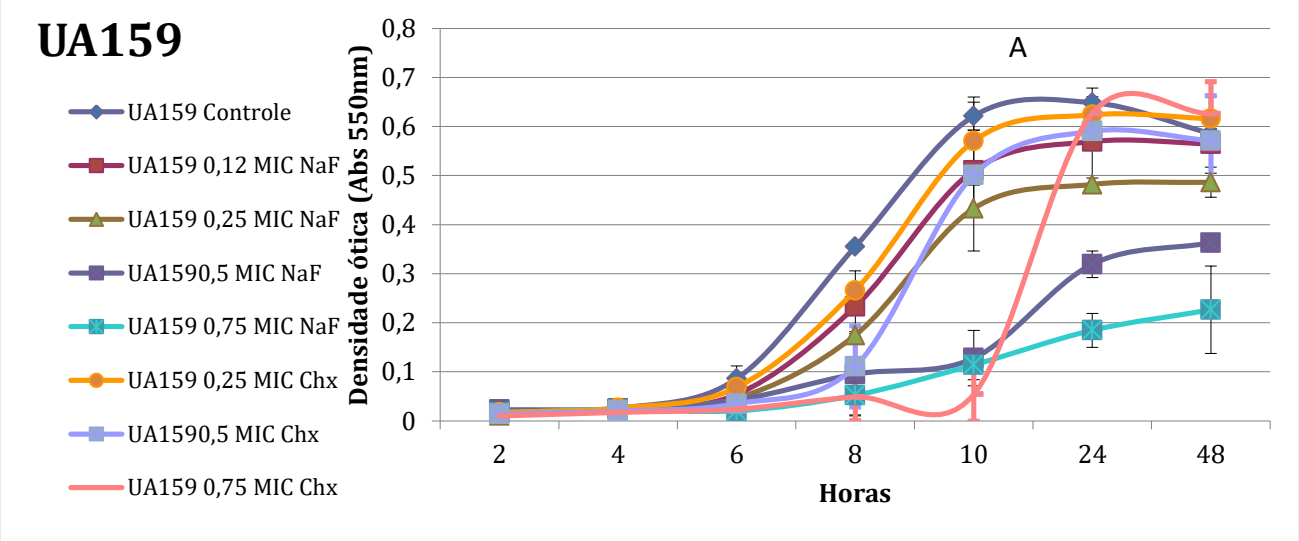

Figura 1A. Curvas de crescimento da cepa de *S. mutans* UA159 exposta ou não aos agentes antimicrobianos.

Para a cepa padrão UA159, houve atraso no crescimento bacteriano a partir da presença de 0,25 x MIC NaF e 0,5 x MIC Chx. Em 10h, a média/desvio padrão (em densidade óptica - DO) de crescimento bacteriano para o grupo controle sem antimicrobianos foi de 0,621±0,02, comparado a 0,433±0,08 para o grupo 0,25 x MIC NaF, 0,128±0,05 para o grupo 0,5 x MIC NaF e 0,114±0,03 para o grupo 0,75 x MIC NaF, demonstrando valores decrescentes no crescimento bacteriano com o aumento da concentração de NaF. Em 48h, a média da DO para o grupo controle manteve-se semelhante (0,585±0,01) quando comparada às 10h, enquanto que para os grupos expostos ao NaF houve um ligeiro aumento no crescimento (grupo 0,25 x MIC - 0,486±0,03, grupo 0,5 x MIC - 0,363±0,005, grupo 0,75 x MIC - 0,226±0,08), de 10h para 24h e se manteve constante e abaixo da média do controle. Em 10h, a média/desvio padrão da DO obtida foi de 0,570 ±0,08 para o grupo 0,25x MIC Chx, 0,501±0,01 para o grupo 0,5 x MIC Chx e 0,054±0,05 para o grupo 0,75 x MIC, notando-se que somente a maior concentração de Chx teve um maior impacto causando atraso no crescimento bacteriano. Após 48h, houve crescimento expressivo da cepa UA159 quando exposta a 0,75 x MIC Chx e as demais mantiveram o crescimento constante.

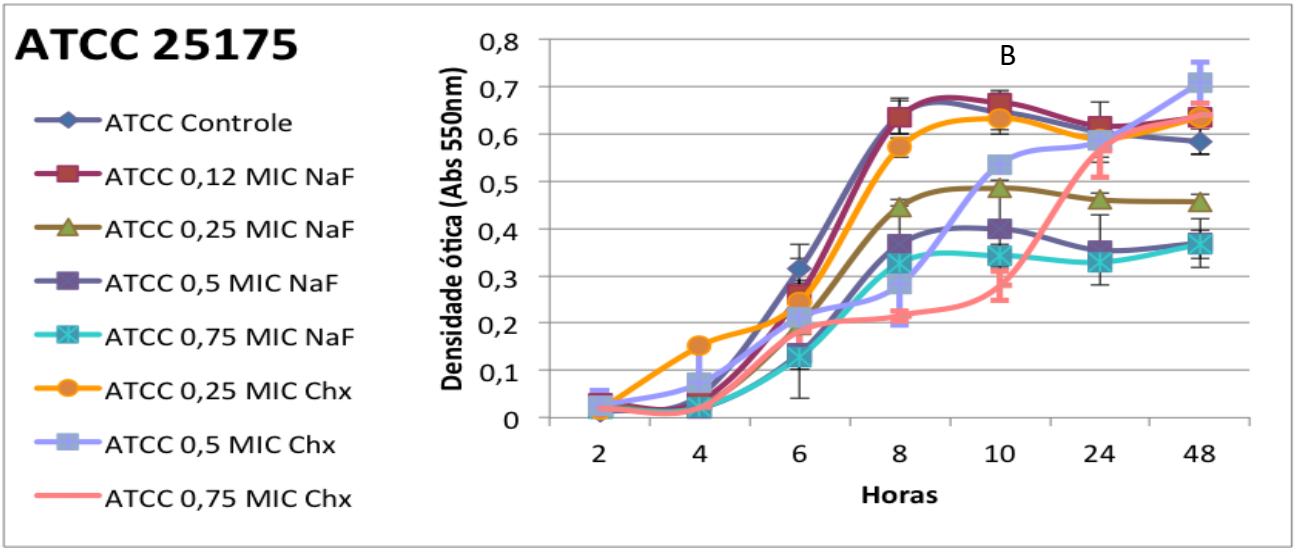

Figura 1B. Curvas de crescimento da cepa de *S. mutans* ATCC 25175 exposta ou não aos agentes antimicrobianos.

Para a cepa *S. mutans* ATCC 25175, houve atraso no crescimento bacteriano a partir da presença de 0,25 x MIC NaF e 0,5 x MIC Chx. Em 8h, a média/desvio padrão (em densidade óptica - DO) de crescimento bacteriano para o grupo controle sem antimicrobianos foi de 0,638±0,03, comparado a 0,445±0,01 para o grupo 0,25 x MIC NaF, 0,366±0,08 para o grupo 0,5 x MIC NaF e 0,327±0,002 para o grupo 0,75 x MIC NaF, apontando para o menor crescimento bacteriano, principalmente após exposição 0,5 x MIC

e 0,75 x MIC NaF. Após 48h, a média da DO para todos os grupos, controle (0,583±0,02) ou expostos a NaF (grupo 0,25 x MIC - 0,456±0,01, grupo 0,5 x MIC - 0,368±0,05, grupo 0,75 x MIC - 0,366±0,03) manteve-se constante quando comparada a 8h. Após 8 h, *S. mutans* ATCC obteve o crescimento minimamente reduzido quando exposto a 0,25 x MIC Chx (0,571±0.02), com maior impacto no crescimento na presença de 0,5 x MIC Chx (0,281±0,08) e 0,75 x MIC (0,215±0,01), entretanto, após 48h, independente da concentração de Chx, o crescimento foi similar entre os grupos expostos e não expostos a Chx.

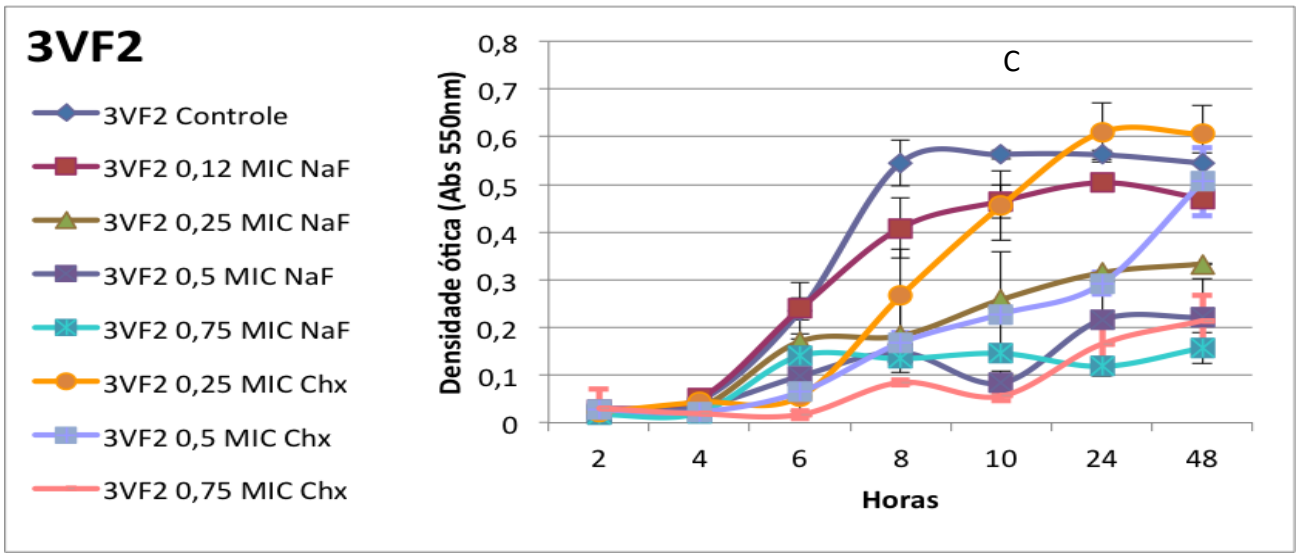

Figura 1C. Curvas de crescimento da cepa de *S. mutans* 3VF2 exposta ou não aos agentes antimicrobianos.

Para a cepa clínica 3VF2, houve atraso no crescimento bacteriano a partir da presença de 0,25 x MIC NaF e 0,25 x MIC Chx. Em 8h, a média/desvio padrão (em densidade óptica - DO) de crescimento bacteriano para o grupo controle sem antimicrobianos foi de 0,545±0,04, comparado a 0,182±0,07 para o grupo 0,25 x MIC NaF, 0,147±0,02 para o grupo 0,5 x MIC NaF e 0,134±0,01 para o grupo 0,75 x MIC NaF, apontando menor crescimento bacteriano quando a cepa foi exposta a NaF, independente das concentrações. Após 48h, a média da DO para o grupo controle (0,544±0,02) se manteve constante e para os grupos expostos a NaF aumentou (grupo 0,25 x MIC - 0,333±0,001, grupo 0,5 x MIC - 0,220±0,08, grupo 0,75 x MIC - 0,156±0,03) quando comparado a 8h, porém manteve-se menor que o controle. Em 8h, as médias de crescimento de 3VF2 foram afetadas na presença de 0,25 x MIC Chx (0,266±0,09), 0,5 x MIC Chx (0,167±0,07) e 0,75 x MIC Chx (0,084±0,04), contudo após 48h, as cepas expostas a 0,25 x MIC (0,605±0,06) e a 0,5 x MIC Chx (0,505±0,07) atingiram taxas de

crescimento similares ao controle, o que não ocorreu com a cepa exposta a 0,75 x MIC Chx (0,214±0,05).

Dentre as cepas avaliadas, quanto ao crescimento planctônico, a cepa clínica 3VF2 foi a mais afetada pelas diferentes concentrações dos agentes antimicrobianos em comparação com as demais cepas de *S. mutans.*

Ensaios de Biofilme

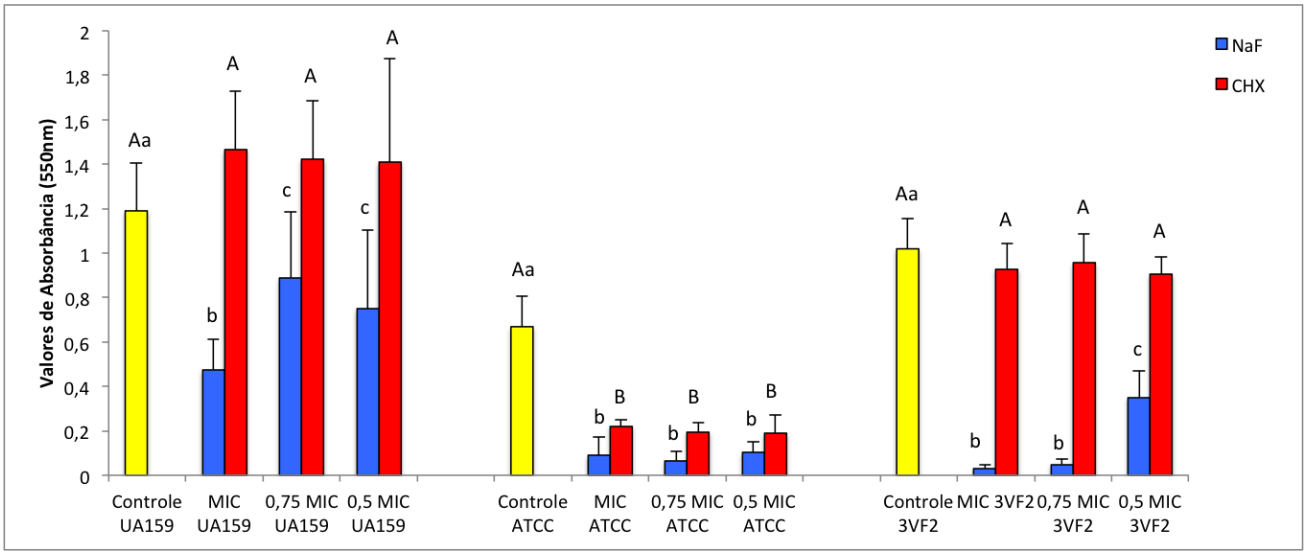

Figura 2. Valores de biomassa do biofilme (Abs – 550nm) obtidos para as cepas de *S. mutans* frente as concentrações subinibitórias de NaF e Chx**.** Barras amarelas- crescimento bacteriano em meio sem nenhum agente antimicrobiano.

[a] Letras minúsculas diferentes mostram diferença estatística entre os grupos contendo NaF, segundo os testes de Kruskal-Wallis/Mann-Whitney.
[A] Letras maiúsculas diferentes mostram diferença estatística entre os grupos contendo Chx, segundo os testes de Kruskal-Wallis/Mann-Whitney.

A **Figura 2** mostra os valores de biomassa de biofilme para as cepas de *S. mutans* avaliadas na presença ou não dos agentes antimicrobianos. A formação de biofilme da cepa clínica 3VF2 e da cepa padrão UA159 não foi afetada quando estas foram expostas às concentrações de 0,5 x MIC, 0,75 x MIC e MIC de Chx. Porém, quando os grupos foram expostos a NaF, obtiveram o crescimento do biofilme reduzido quando comparados ao controle sem NaF. Houve diferença estatística quanto à formação de biofilme entre as concentrações de MIC e as demais sub-MIC quanto à formação de biofilme para a cepa UA159 e entre MIC e 0,75 x MIC comparados a 0,5 x MIC para 3VF2. Todas as concentrações testadas de NaF e Chx afetaram o crescimento da cepa padrão ATCC

25175 quando comparada ao controle sem os antimicrobianos, sem diferença estatística entre as concentrações.

Análise da expressão de *covR* e *vicR* de *S. mutans*

A **Tabela 2** mostram os resultados obtidos para a expressão dos genes *covR e vicR* para as demais cepas de *S .mutans* avaliadas no estudo.

Tabela 2. Médias (desvios-padrão) dos valores (em ng/mL) das concentrações dos cDNA obtidos para os genes *covR e vicR* das cepas de *S. mutans,* normalizados pelo gene 16SRNA. Valores em folds (desvio-padrão) nos genes *covR e vicR* foram expressos comparando-se as diferentes concentrações de Chx ou NaF ou Chx x NaF .

GRUPOS Cepa/[ ] em g/mL	Média (dp) – ng/mL *covR*	Média (dp) – ng/mL *vicR*
UA159 0,125 x MIC Chx	1.49 (0.09)	1.37 (0.18)
UA159 0,25 x MIC Chx	1.42 (0.2)	1.05 (0.19)
UA159 0,5 x MIC Chx	1.28 (0.4)	1.21 (0.32)
ATCC 0,125 x MIC Chx	1.58 (0.38)	1.29 (0.02)
ATCC 0,25 x MIC Chx	1.00 (0.43)	1.15 (0.10)
ATCC 0,5 x MIC Chx	1.42 (0.15)	1.62 (0.09)
3VF2 0,125 x MIC Chx	1.71 (0.11)	1.5 (0.27)
3VF2 0,25 x MIC Chx	1.37 (0.19)	0.85 (0.06)
3VF2 0,5 x MIC Chx	2.65 (0.5)	0.91 (0.11)
UA159 0,125 x MIC NaF	1.52 (0.41)	1.35 (0.19)
UA159 0,25 x MIC NaF	1.05 (0.13)	1.11 (0.32)
UA159 0,5 x MIC NaF	1.36 (0.56)	1.19 (0.48)
ATCC 0,125 x MIC NaF	0.50 (0.19)	0.50 (0.31)
ATCC 0,25 x MIC NaF	0.51 (0.28)	0.73 (0.37)
ATCC 0,5 x MIC NaF	0.42 (0.29)	0.57 (0.34)
3VF2 0,125 x MIC NaF	1.14 (0.56)	0.86 (0.2)
3VF2 0,25 x MIC NaF	0.70 (0.43)	0.65 (0.1)
3VF2 0,5 x MIC NaF	0.43 (0.31)	0.61 (0.5)

Cepa/Gene	[ ]/[ ] em folds 0,25 x MIC /0,125 x MIC	[ ]/[ ] em folds 0,5 x MIC/0,125 x MIC	[ ]/[ ] em folds 0,5 x MIC/0,25 x MIC
Chx			
UA159/*covR*	0.54 (0.05)	0.85 (0.32)	0.84 (0.22)
3VF2/*covR*	0.85 (0.12)	1.58 (0.25) ‡	1.85 (0.08) ‡
ATCC/*covR*	0.85 (0.15)	0.84 (0.19)	1.00 (0.23)
UA159/*vicR*	0.76 (0.05) †	0.86 (0.23) †	1.14 (0.32) †
3VF2/*vicR*	0.92 (0.07) †	1.26 (0.06) ‡	1.36 (0.15) ‡
ATCC/*vicR*	0.54 (0.05) ‡	0.57 (0.06) ‡	0.57 (0.06)
NaF			
UA159/*covR*	0.70 (0.17) †	0.91 (0.12)	1.37 (0.45) †
3VF2/*covR*	0.60 (0.12) †	0.37 (0.07)	0.64 (0.2) †
ATCC/*covR*	0.84 (0.35)	0.94 (0.34)	1.21 (0.5)
UA159/*vicR*	0.85 (0.13)	0.86 (0.30)	0.99 (0.26)
3VF2/*vicR*	0.74 (0.2)	0.51 (0.36)	0.78 (0.56)
ATCC/*vicR*	0.99 (0.3)	1.17 (0.28) †	1.29 (0.53) †
NaF/Chx	**0,125 x MIC**	**0,25 x MIC**	**0,5 x MIC**
UA159 covR	2.5 (0.6) ‡	2.4 (0.5) ‡	1.95 (0.72)
UA159 vicR	1.02 (0.08)	0.94 (0.22)	1.08 (0.31)
3VF2 covR	1.24 (0.61)	1.79 (0.5) †	4.7 (2.0) ‡
3VF2 vicR	1.2 (0.43)	1.71 (0.1) †	2.17 (0.63) †
ATCC covR	2.5 (0.6) ‡	2.0 (0.35) ‡	1.85 (0.23) †
ATCC vicR	2.27 (0.17) †	1.07 (0.26)	1.09 (0.1)

‡ Diferença estatística entre os grupos discriminados na primeira coluna comparando-se os valores em ng/mL, considerando as três condições testadas: Chx, NaF e NaFxChx, considerando p<0,01, de acordo com os testes de ANOVA/Tukey. † Diferença estatística entre os grupos discriminados na primeira coluna comparando-se os valores em ng/mL, considerando as três condições testadas: Chx, NaF e NaFxChx, considerando p<0,05, de acordo com os testes de ANOVA/Tukey.

De forma geral, o gene *vicR* foi mais afetado pela presença de concentrações subinibitórias de Chx, considerando as três situações testadas: 0,25 x MIC/0,125 x MIC ou 0,5 x MIC/0,125 x MIC ou 0,5 x MIC/0,25 x MIC, aumentando sua expressão para as cepas UA159, 3VF2 e em menor escala para ATCC (exceto para 0,5 x MIC/0,25 x MIC). Ainda considerando a Chx, para o gene *covR* somente a cepa 3VF2 foi afetada quando comparando 0,5 x MIC/0,125 x MIC ou 0,5 x MIC/0,25 x MIC com 0,25 x MIC/0,125 x MIC, aumentando sua expressão. Para NaF, o gene *covR* teve um aumento de sua expressão quando comparado 0,25 x MIC/0,125 x MIC ou 0,5 x MIC/0,25 x MIC com 0,5 x MIC/0,125 x MIC para UA159 e 3VF2. NaF aumentou a expressão de *vicR* para a cepa ATCC somente quando comparados 0,5 x MIC/0,125 x MIC ou 0,5 x MIC/0,25 x MIC com 0,25 x MIC/0,125 x MIC. Quando NaF e Chx foram relacionados, considerando a mesma condição de concentração subinibitória, para a cepa UA159, o gene *covR* obteve sua expressão reduzida para a maior concentração 0,5 x MIC comparando-se com as menores concentrações 0,125 x MIC ou 0,25 x MIC. Para *vicR* não foi verificada diferença entre as concentrações. Para a cepa 3VF2, ambos os genes obtiveram sua expressão aumentada quando comparados NaF e Chx para as concentrações de 0,25 x MIC e 0,5 x MIC de ambos os antimicrobianos comparando-se com 0,125 x MIC. Para ATCC, a expressão do gene *covR* diferiu entre as três concentrações avaliadas (0,125, 025 e 0,5), sendo menor em 0,5 x MIC e a expressão do gene *vicR* foi superior na concentração 0,125 x MIC quando comparada às demais concentrações.

DISCUSSÃO

Streptococcus mutans tem sido associado a etiologia da cárie dentária devido a sua capacidade de adesão e colonização da estrutura dentária. Agentes anticárie, tais como a clorexidina (Chx) e o fluoreto de sódio (NaF), são amplamente estudados com o intuito de prevenir a cárie dentária pela inibição da formação de biofilme. Entretanto, as concentrações subinibitórias desses produtos poderiam induzir a expressão de alguns genes relacionados com a formação de biofilme e aumentar a colonização dessas espécies na cavidade bucal [23]. Diversos sistemas de transdução de sinais são ativados em bactérias em resposta a alterações no microambiente regulando a expressão de genes frente a esses estímulos.

Neste estudo, cepas de *S. mutans* analisadas tiveram seu crescimento afetado a partir da exposição de 0,25 x MIC NaF para todas as cepas testadas e 0,25 x MIC para 3VF2 e 0,5 x MIC Chx para UA159 e ATCC 25175. Dong et. al. (23) determinaram os valores de MIC/MBC de NaF e Chx para *S. mutans* UA159 a fim de expor essa cepa a concentrações subinibitórias desses agentes. Embora os resultados de MIC/MBC deste trabalho tenha diferido do presente estudo (NaF – MIC = 0,625µg/mL e MBC = 2,5 µg/mL; Chx – MIC – 2,5 µg/mL e MBC=2,5 µg/mL), os valores de DO para as curvas de crescimento foram semelhantes, sendo que 0,5 x MIC de NaF interferiu drasticamente no crescimento de *S.mutans* e 0,5 x MIC Chx permitiu o crescimento com atraso em relação ao controle sem a presença dos antimicrobianos. Resultados similares foram encontrados para as cepas de *S. mutans* avaliadas no presente estudo. Níveis sub-MIC de agentes antimicrobianos não apresentam ação bactericida, mas mostram ter um efeito inibitório na fisiologia bacteriana [33].

Os mecanismos de ação do flúor sobre microrganismos ainda estão sendo elucidados. O flúor penetra na forma de HF devido a uma diferença de pH entre o meio extracelular mais ácido que o meio intracelular. No meio intracelular, o HF se dissocia em íons hidrogênio (H+) e flúor (F-), e o acúmulo de prótons acidifica o citoplasma, reduzindo o gradiente de prótons e consequentemente todo o metabolismo da bactéria [8, 9, 34]. O excesso de prótons altera a permeabilidade celular, pois inviabiliza as proteínas de membrana, ou seja, reduz a capacidade da célula de transportar nutriente,

inclusive açúcares, que seriam transportados para o seu interior através de uma proteína carregadora, contra o gradiente de concentração, ou seja, com gasto de ATP. Além disso, o flúor inibe a enolase, enzima da via glicolítica que converte 2-fosfoglicerato a fosfoenolpiruvato, causando redução do metabolismo de glicose, e consequentemente diminui a síntese de ATP. Isso descompensa ainda mais o pH, pois ocorre a inibição da bomba translocadora de prótons responsável pela liberação do excesso de íons H^+ intracelular. Esse desarranjo bioquímico culmina na morte bacteriana [9, 34].

A capacidade de formação de biofilme é uma das habilidades que torna *S. mutans* um dos microrganismos mais persistentes na colonização da cavidade bucal. As células no biofilme diferem em suas funcionalidades quando comparadas com aquelas em condição planctônica [35]. Redução no volume total do biofilme e na expressão dos genes *gtfB, gtfC e gtfD* de *S. mutans* foi observada quando biofilmes de *S. mutans* foram expostos a sub-MIC (100 µg/ml) de NaF [33]. A menor produção de GTFs poderia interferir na síntese de EPS, reduzindo a capacidade de aderência da bactéria na superfície dental e consequente formação de biofilme. Neste estudo, concentrações inibitórias e subinibitórias de NaF reduziram a formação de biofilme para todas as cepas testadas, atuando de maneira dose-dependente para as cepas 3VF2 e UA159. Mínimas concentrações de NaF afetaram drasticamente a formação de biofilme da cepa *S. mutans* ATCC 25175 possivelmente por sua menor habilidade de formar biofilme em comparação com as demais cepas testadas.

A presença de agentes antimicrobianos na cavidade bucal é considerada um fator de estresse para *S. mutans* e atua como estímulo para a regulação de genes alvo ou sistemas gênicos, como o sistema de dois componentes (TCS) que tem se mostrado essenciais para a adaptação, sobrevivência e virulência bacteriana [29]. Em espécies de estreptococos, o sistema vicRK se apresenta em forma de operon formado por três cístrons (parte do gene que contém as informações para a síntese de uma proteína) – vicR, vicK e vicX. Os dois primeiros codificam o regulador de resposta (VicR) e a proteína sensora (VicK) e os demais componentes codificam proteínas consideradas acessórias ao sistema [36]. O sistema vicRK participa da ativação de genes essenciais para a competência genética, formação de biofilme, adesão mediada por sacarose, resistência ao estresse oxidativo, produção e tolerância ácida, produção de

bacteriocinas e metabolismo de parede celular em *S. mutans* [1, 26, 28, 37, 38]. A regulação do sistema VicRK interfere na expressão dos genes *gtfB, gtfC, gtfD, ftf* (frutosiltransfares) *e gbpB* que atuam, entre outras funções, na formação do biofilme [26, 39]. Neste presente estudo, concentrações subinibitórias de Chx aumentaram a expressão de *vicR*, em concentração dose-dependente, para as cepas de *S. mutans* testadas. O efeito da Chx sobre bactérias ainda não está completamente elucidado. Contudo, é conhecido que Chx por ser catiônica interage com resíduos de fosfato aniônico das moléculas lipídicas na membrana celular por adsorção. A Chx também atravessa a membrana plasmática e precipita o conteúdo citoplasmático através da formação de complexos com os fosfatos [40]. Chx também induz a formação de "dented spots" (tipo de poro) na parede celular das bactérias tanto Gram-positivas quanto Gram-negativas, porém com diferente localização, dependendo do Gram [40]. O sistema VicRK também é conhecido por regular genes relacionados com propriedades da superfície celular de *S. mutans*, como *gbpB* [28]. Sendo assim, possivelmente Chx está estimulando o sistema VicRK e este induzindo a produção de enzimas duplamente relacionadas com a função de formação de biofilme, pois mesmo concentrações sub-MIC mais elevadas de Chx não influenciaram negativamente na formação do biofilme e também de metabolismo da parede celular. Assim, outros genes-chave, como *gbpB*, poderiam ser analisados para auxiliar na elucidação desse mecanismo.

Outro sistema TCS bastante estudado é o CovRS que é composto por dois cístrons: *covR*, codificador do regulador de resposta CovR, e *covS*, codificador da proteína sensora CovS. Diferente da maioria dos sistemas TCS, CovR atua como repressor de transcrição dos genes diretamente por ele regulados [41]. Neste estudo, a expressão de *covR* foi reduzida na maior concentração de sub-MIC (0,5 x MIC) quando NaF e Chx foram relacionados (NaF/Chx) para a cepas de *S. mutans* UA159 e ATCC. O mesmo não foi observado para a cepa 3VF2. Coincidentemente, nesta concentração (0,5 x MIC), ambas as cepas UA159 e ATCC apresentaram maior resistência a Chx e NaF e consequentemente maior crescimento em condição planctônica que a cepa clínica 3VF2. É conhecido que o sistema VicRK induz a expressão de gtfB/C/D e gbpB [26] enquanto que CovR reprime a expressão de, pelo menos, gtfB/C/D e gbpC [42-44]. Stipp et al. [29] demonstraram grande produção de

biofilme em cepas knockout do gene covR e produção 3x maior de GbpB pela cepa UA159. Mais estudos são necessários para confirmar os mecanismos de resposta de *S. mutans* frente concentrações subinibitórias de Chx e de NaF.

CONCLUSÕES

1) Concentrações subinibitórias de Chx e de NaF afetaram o crescimento planctônico das cepas de *S. mutans*, de maneira dose-dependente.
2) Concentrações subinibitórias de Chx não afetaram a formação de biofilme das cepas de *S. mutans* UA159 e 3VF2, somente da cepa padrão ATCC 25175.
3) Concentrações subinibitórias de NaF acima de 0,5 x MIC causaram redução do crescimento do biofilme para todas as cepas de *S. mutans* testadas, de maneira dose-dependente, com exceção da cepa padrão ATCC 25175, que foi afetada igualmente por todas as concentrações avaliadas.
4) Concentrações subinibitórias gradativas de Chx induziram a expressão crescente do gene *vicR* das cepas de *S. mutans* avaliadas.
5) Concentrações subinibitórias de NaF e Chx estimularam a expressão dos genes *covR e vicR*, entretanto, houve uma grande variação na expressão gênica de acordo com a cepa de *S. mutans* e condições de exposição aos agentes antimicrobianos testadas.

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ANEXO 1

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File format	Manuscript files can be in the following formats: DOC, DOCX, RTF, or PDF. Microsoft Word documents should not be locked or protected. LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.
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Use correct and established nomenclature wherever possible.

Units of measurement	Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.
Drugs	Provide the Recommended International Non-Proprietary Name (rINN).
Species names	Write in italics (e.g., **Homo sapiens**). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., **H. sapiens**).
Genes, mutations, genotypes, and alleles	Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HUGO for human genes). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC).

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- Introduction

Middle section

The following elements can be renamed as needed and presented in any order:

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- Results
- Discussion
- Conclusions (optional)

Ending section

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Short title	100 characters	State the topic of the study	Cigarette Smoke Exposure and Innate Immunity SODIS and Childhood Diarrhoea

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The submitting author is automatically designated as the corresponding author in the submission system. The corresponding author is the primary contact for the journal office and the only author able to view or change the manuscript while it is under editorial consideration.

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Only one corresponding author can be designated in the submission system, but this does not restrict the number of corresponding authors that may be listed on the article in the event of publication. Whoever is designated as a corresponding author on the title page of the manuscript file will be listed as such upon publication. Include an email address for each corresponding author listed on the title page of the manuscript.

 **How to select a new corresponding author in Editorial Manager**

Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include the consortium or group name in the author list, and include the full list of members in the Acknowledgments or in a supporting information file. Read the group authorship policy.

Author Contributions

Enter all author contributions in the submission system during submission. The contributions of all authors must be described using the CRediT Taxonomy of author roles. Read the policy.

Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and it is expected that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time.

PLOS ONE will contact all authors by email at submission to ensure that they are aware of the submission.

Cover letter

Upload a cover letter as a separate file in the online system. The length limit is 1 page.

The cover letter should include the following information:

- Summarize the study's contribution to the scientific literature
- Relate the study to previously published work
- Specify the type of article (for example, research article, systematic review, meta-analysis, clinical trial)
- Describe any prior interactions with PLOS regarding the submitted manuscript
- Suggest appropriate Academic Editors to handle your manuscript (see the full list of Academic Editors)
- List any opposed reviewers

IMPORTANT: Do not include requests to reduce or waive publication fees in the cover letter. This information will be entered separately in the online submission system.

Read about publication fee assistance.

Title page

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

Download sample title, author list, and affiliations page (PDF)

Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
- Abbreviations, if possible

Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

We encourage authors to submit detailed protocols for newer or less well-established methods as supporting information. Read the supporting information guidelines.

Human or animal subjects and/or tissue or field sampling

Methods sections describing research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the reporting guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

Data

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

Large data sets, including raw data, may be deposited in an appropriate public repository. See our list of recommended repositories.

For smaller data sets and certain data types, authors may provide their data within supporting information files accompanying the manuscript. Authors should take care to maximize the accessibility and reusability of the data by selecting a file format from which data can be efficiently extracted (for example, spreadsheets or flat files should be provided rather than PDFs when providing tabulated data).

For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to "data not shown."

Cell lines

Methods sections describing research using cell lines must state the origin of the cell lines used. See the reporting guidelines for cell line research for more information.

New taxon names

Methods sections of manuscripts adding new taxon names to the literature must follow the reporting guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Criteria for Publication for more information.

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, if the manuscript is submitted to a journal and also publicly available as a preprint

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., “We used the techniques developed by our colleagues [19] to analyze the data”). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts or author summaries.

Make sure the parts of the manuscript are in the correct order **before** ordering the citations.

Formatting references

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the “Vancouver” style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source	Format
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (**Ailuropoda melanoleuca**). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005 **Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.**
Accepted, unpublished articles	Same as published articles, but substitute “Forthcoming” for page numbers or DOI.
Web sites or online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14.
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available from: arXiv:1403.3301v1. Cited 17 March 2014.
Published media (print or online	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available from:

Source	Format
newspapers and magazine articles)	http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html. Cited 17 March 2014.
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214.
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

Supporting Information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 10 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an “S” and number. For example, “S1 Appendix” and “S2 Appendix,” “S1 Table” and “S2 Table,” and so forth.

Supporting information files are published exactly as provided, and are not copyedited.

Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

Figures and Tables

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order upon first appearance in the manuscript file.

Read the guidelines for figures.

Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- A figure label with Arabic numerals, and “Figure” abbreviated to “Fig” (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of “Fig 1” must refer to a figure file named “Fig1.tif”).
- A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

Tables

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Tables require a label (e.g., “Table 1”) and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Read the guidelines for tables.

Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

Read our policy on data availability.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

See our list of recommended repositories.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers of DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at submission.

Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

Striking image

You can choose to upload a “Striking Image” that we may use to represent your article online in places like the journal homepage or in search results.

The striking image must be derived from a figure or supporting information file from the submission, i.e., a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows.

If no striking image is uploaded, we will designate a figure from the submission as the striking image.

Striking images should not contain potentially identifying images of people. Read our policy on identifying information.

The PLOS licenses and copyright policy also applies to striking images.

Additional Information Requested at Submission

Funding statement

This information should not be in your manuscript file; you will provide it via our submission system.

This information will be published with the final manuscript, if accepted, so please make sure that this is accurate and as detailed as possible. You should not include this information in your manuscript file, but it is important to gather it prior to submission, because your financial disclosure statement cannot be changed after initial submission**.**

Your statement should include relevant grant numbers and the URL of any funder's web site. Please also state whether any individuals employed or contracted by the funders (other than the named authors) played any role in: study design, data collection and analysis, decision to publish, or preparation of the manuscript. If so, please name the individual and describe their role.

Read our policy on disclosure of funding sources.

Competing interests

This information should not be in your manuscript file; you will provide it via our submission system.

All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full.

Read our policy on competing interests.

Manuscripts disputing published work

For manuscripts disputing previously published work, it is **PLOS ONE** policy to invite input from the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process.

If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.

Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

Related manuscripts

Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to **PLOS ONE** or elsewhere, authors must include a copy with the submitted article. Reviewers will be asked to comment on the overlap between related submissions.

We strongly discourage the unnecessary division of related work into separate manuscripts, and we will not consider manuscripts that are divided into "parts." Each submission to **PLOS ONE** must be written as an independent unit and should not rely on any work that has not already been accepted for publication. If related manuscripts are submitted to **PLOS ONE**, the authors may be advised to combine them into a single manuscript at the editor's discretion.

PLOS does support authors who wish to share their work early and receive feedback before formal peer review. Deposition of manuscripts with preprint servers does not impact consideration of the manuscript at any PLOS journal.

Authors choosing bioRxiv may now concurrently submit directly to select PLOS journals through bioRxiv's direct transfer to journal service.

Read our policy on preprint servers.

Guidelines for Specific Study Types

Human subjects research

All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or by equivalent ethics committee(s), and must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should be able to submit, upon request, a statement from the IRB or ethics committee indicating approval of the research. We reserve the right to reject work that we believe has not been conducted to a high ethical standard, even when formal approval has been obtained.

Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). Download additional translations of the form from the Downloads and Translations page. More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

Manuscripts should conform to the following reporting guidelines:

- Studies of diagnostic accuracy: STARD
- Observational studies: STROBE
- Microarray experiments: MIAME

- Other types of health-related research: Consult the EQUATOR web site for appropriate reporting guidelines

Methods sections of papers on research using human subjects or samples must include ethics statements that specify:

- **The name of the approving institutional review board or equivalent committee(s)**. If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- **Whether informed consent was written or oral**. If informed consent was oral, it must be stated in the manuscript:
    - Why written consent could not be obtained
    - That the Institutional Review Board (IRB) approved use of oral consent
    - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: "Caucasian" should be changed to "white" or "of [Western] European descent" (as appropriate); "cancer victims" should be changed to "patients with cancer."

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal, which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about **PLOS ONE** policies regarding human subjects research, see the Publication Criteria and Editorial Policies.

Clinical trials

Clinical trials are subject to all policies regarding human research. **PLOS ONE** follows the World Health Organization's (WHO) definition of a clinical trial:

A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

PLOS ONE supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. **Where trials were not publicly registered before participant recruitment began**, authors must:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- Provide the registry name and number in the methods section of the manuscript
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's “Fig 1”

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

Animal research

We work in consultation with the **PLOS ONE** Animal Research Advisory Group to develop policies. Animal Research Advisory Group members may also be consulted on individual submissions.

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.

If we note differences between an IACUC-approved protocol and the methods reported in a submitted manuscript, we may report these discrepancies to the relevant institution or committee.

Methods sections of manuscripts reporting results of animal research must include required ethics statements that specify:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s). Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why.
- Relevant details for efforts taken to ameliorate animal suffering

Example ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

The organism(s) studied should always be stated in the abstract. Where research may be confused as pertaining to clinical research, the animal model should also be stated in the title.

Where unregulated animals are used or ethics approval is not required, authors should make this clear in submitted articles and explain why ethical approval was not required. Relevant regulations that grant exemptions should be cited in

full. It is the authors' responsibility to understand and comply with all relevant regulations.

We reserve the right to reject work that the editors believe has not been conducted to a high ethical standard, even if authors have obtained formal approval or approval is not required under local regulations.

We encourage authors to follow the Animal Research: Reporting of **In Vivo** Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information. Please note that inclusion of a completed ARRIVE Checklist may be a formal requirement for publication at a later date.

Non-human primates

Manuscripts describing research involving non-human primates must include details of animal welfare, including information about housing, feeding, and environmental enrichment, and steps taken to minimize suffering, including use of anesthesia and method of sacrifice if appropriate, in accordance with the recommendations of the Weatherall report, **The use of non-human primates in research** (PDF).

Humane endpoints

For studies in which death of a regulated animal (vertebrate, cephalopod) is a likely outcome or a planned experimental endpoint, **PLOS ONE** asks authors to report additional details related to the study design. This applies to research that involves, for instance, assessment of survival, toxicity, longevity, terminal disease, or high rates of incidental mortality. These studies may be subject to additional ethical considerations, and **PLOS ONE** may reject submissions if they lack sufficient reporting, appropriate justification for the study design, or adequate consideration of humane endpoints, regardless of study-specific institutional animal ethics committee approval.

Definition of a humane endpoint

A humane endpoint is an experimental endpoint at which animals are euthanized when they display early markers associated with death or poor prognosis of quality of life, or specific signs of severe suffering or distress. Humane endpoints are used as an alternative to allowing such conditions to continue or progress to death following the experimental intervention (“death as an endpoint”), or only euthanizing animals at the end of an experiment. Before a study begins, researchers define the practical observations or measurements that will be used during the study to recognize a humane endpoint, based on anticipated clinical, physiological, and behavioral signs. These may include, for instance, body temperature or weight changes, tumor size or appearance, abnormal behaviors, pathological changes, ruffled fur, reduced mobility, body posture, or expression of specific body fluid markers. Please see the NC3Rs guidelines for more information.

Authors of these studies should report all of the following information in the Methods section:

1. Describe whether humane endpoints were used for all animals involved in the study

If humane endpoints were used, report the following:

- The specific criteria used to determine when animals should be euthanized
- Once animals reached endpoint criteria, the amount of time elapsed before euthanasia
- Whether any animals died before meeting criteria for euthanasia

If humane endpoints were not used, report the following:

- A scientific and ethical justification for the study design, including the reasons why humane endpoints could not be used, and discussion of alternatives that were considered but could not be used
- Whether the institutional animal ethics committee specifically reviewed and approved the anticipated mortality in the study design

2. Include the following details of the study design and outcomes:

- The duration of the experiment
- The numbers of animals used, euthanized, and found dead (if any); the cause of death for all animals
- How frequently animal health and behavior were monitored
- All animal welfare considerations taken, including efforts to minimize suffering and distress, use of analgesics or anaesthetics, or special housing conditions
- Any special training in animal care or handling provided for research staff

Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- **Sharing of data and materials.** Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under **PLOS ONE**'s data availability criterion.
- **Ethics. PLOS ONE** will not publish research on specimens that were obtained without necessary permission or were illegally exported

Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as supporting information

Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo **et al.**

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section.

Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants' experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent.

For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research.
See our reporting guidelines for human subjects research.

Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

For de novo **(new) cell lines**, including those given to the researchers a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

Blots and gels

Manuscripts reporting results from blots (including Western blots) and electrophoretic gels should follow these guidelines:

- In accordance with our policy on image manipulation, the image should not be adjusted in any way that could affect the scientific information displayed, e.g. by modifying the background or contrast.
- All blots and gels that support results reported in the manuscript should be provided.
- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files.
- Lanes should not be overcropped around the bands; the image should show most or all of the blot or gel. Any non-specific bands should be shown and an explanation of their nature should be given.
- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples.
- A figure panel should not include composite images of bands originating from different blots or gels. If the figure shows non-adjacent bands from the same blot or gel, this should be clearly denoted by vertical black lines and the figure legend should provide details of how the figure was made.

Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species.
- The commercial supplier or source laboratory.
- The catalogue or clone number and, if known, the batch number.
- The antigen(s) used to raise the antibody.
- For established antibodies, a stable public identifier from the Antibody Registry.

The manuscript should also report the following experimental details:

- The final antibody concentration or dilution.
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

We encourage authors to consider adding information on new validations to a publicly available database such as Antibodypedia or CiteAb.

Methods, software, databases, and tools

PLOS ONE will consider submissions that present new methods, software, or databases as the primary focus of the manuscript if they meet the following criteria:

Utility
The tool must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online tools, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

Validation
Submissions presenting methods, software, databases, or tools must demonstrate that the new tool achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new tool is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

Availability
Software should be open source, deposited in an appropriate archive, and conform to the Open Source Definition. Databases must be open-access and hosted somewhere publicly accessible, and any software used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. Authors should provide a direct link to the deposited software or the database hosting site from within the paper.

Software submissions

Manuscripts describing software should provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

New taxon names

Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

***Anochetus boltoni* Fisher *sp. nov.* urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB**

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the **Methods** section, in a sub-section to be called “Nomenclatural Acts”:

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend

that you also deposit the published online article there and include the name in your article.

Botanical names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Solanum aspersum S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

Journal staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are

effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Fungal names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Hymenogaster huthii. Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]

You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Qualitative research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.