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Effects of *Trigona* honey on the Gene Expression Profile of *Pseudomonas aeruginosa* ATCC 10145 and *Streptococcus pyogenes* ATCC 19615

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Abstract

Honey is a broad-spectrum antimicrobial agent that seems to affect different bacteria in many different ways. The aim of this study was to evaluate the antibacterial activities of *Trigona* honey against *P. aeruginosa* and *S. pyogenes*. The effect of *Trigona* honey on *P. aeruginosa* and *S. pyogenes* was investigated using growth kinetics curve and real-time PCR. The growth kinetics of *P. aeruginosa* and *S. pyogenes* with 20% (w/v)(MIC) *Trigona* honey inhibited the growth cells number of *P. aeruginosa* and *S. pyogenes* compared with cells grown without honey. Treatment with 10% (w/v) (half-MIC) showed slightly decreased number of cells of *P. aeruginosa* and *S. pyogenes* over a period of 24 hours. Conversely, treatment with 5% (w/v) (quarter-MIC) was observed to have a similar untreated samples of *P. aeruginosa* and *S. pyogenes*. The RT-qPCR results showed that the expression of *Sof* and *Sfbl* decreased 7.82-fold and 9.23-fold respectively after exposure to 20% concentration of *Trigona* honey, whereas the expression of *algD* and *oprF* decreased 6.28-fold and 11.11-fold respectively after exposure to 20% concentration of *Trigona* honey. *Trigona* honey demonstrated the highest antibacterial activity against *P. aeruginosa* and *S. pyogenes in vitro*. Our results indicate that *Trigona* honey has the potential to be an effective inhibitor on virulence genes of *P. aeruginosa* and *S. pyogenes*.

Keywords: Pseudomonas aeruginosa, Streptococcus pyogenes, Trigona honey, Growth kinetics, RT-qPCR.

1. Introduction

Pseudomonas aeruginosa is a pathogen of plants, animals and humans with colonies as diverse as the site of isolation and is able to cause devastating infections because of the strong attachment potential with pili (Lyczak *et al*., 2000; Van Delden and Iglewski, 1998) and production multiple virulence genes such as *oprF* and *algD* (van Delden, 2004; Wendt *et al*., 2017). *oprF* is a general outer membrane porin of *P.aeruginosa* allowing nonspecific diffusion of ionic species and small polar nutrients, including polysaccharides (Nestorovich *et al*., 2006). *oprF* is described as a structural protein, anchoring the outer membrane to the peptidoglycan layer (Rawling *et al*., 1998; Woodruff and Hancock, 1989). The structure and positioning of *oprF* provides structural stability, forming an anchor point between the outer membrane and peptidoglycan layer (Bouffartigues *et al*., 2015; Chevalier *et al*., 2017; Fito-Boncompte *et al*., 2011; Rosay *et al*., 2015; Sugawara *et al*., 2006). The reduced expression of this gene not only could result in the disruption of diffusion, but may also compromise cellular integrity; the latter supports the scanning microscopy data, which showed a loss and disruption of extracellular components and abnormal cell structure observed in previous studies (Henriques *et al*., 2011; Al-Kafaween *et al*., 2019). Currently, *oprF* has been shown to play a role in the growth and biofilm formation (Bouffartigues *et al*., 2015; Chevalier *et al*., 2017; Fito-Boncompte *et al*., 2011; Rosay *et al*., 2015; Sugawara *et al*., 2006) of *P.aeruginosa* (Bjarnsholt *et al*., 2008; James *et al*., 2008; Yoon *et al*., 2002). The mucoid phenotype of *P.aeruginosa* is caused by excessive alginate production and has long been associated with prolonged infection of the pulmonary cavity, particularly in those suffering from cystic fibrosis (Jones *et al*., 2013; McIntyre *et al*., 2010; Wozniak *et al*., 2003). Recent studies (Wood and Ohman, 2012) have shown that alginate genes are under the regulation of sigma factor (s22) and are upregulated as part of an extra cytoplasmic stress response to cell wall stress. Of the many genes regulated, 11 are directly involved in cell envelope homeostasis. The *algD* (GDP-mannose dehydrogenase), which is essential for the production of alginate (*algB*, alg*C*, *algE*, *algR*, *algG* and *algT*) and as a virulence factor (Leid *et al*., 2005; May *et al*., 1991). Additionally, *algD* is

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a key enzyme of the alginate biosynthetic pathway (Manzo et al., 2011;Jones *et al*., 2013; McIntyre *et al*., 2010; Wozniak *et al*., 2003). *S. pyogenes* produces a wide range of virulence factors, and the cell wall associated M protein is a major virulence factor of *S. pyogenes*, which can bind directly to the extracellular matrix components (e.g. fibrinogen)(Cole *et al*., 2007; Yamaguchi *et al*., 2013). Fibronectin (Fn) is a high-molecular weight glycoprotein that circulates free as a dimer in the soluble form in blood plasma or as a fibrillar form is assembled by cells as major component of the extracellular matrix. So far, fibronectin binding proteins are the best studied adhesions of *S. pyogenes* and currently 11 different such adhesions have been identified (Yamaguchi *et al*., 2013), divided in two types. First type proteins are SfbI, PrtF2, Sof, SfbX, Fbp54, FbaA, and FbaB and they all contain Fn-binding repeats. Second type proteins are M1, Shr, Scl1, and GAPDH and they do not contain these repeats. It is estimated that 60% of initial attachment to cells is realized by streptococcal lipoteichoic acid. Fibronectin binding proteins are the most important in the irreversible stage of adherence. Binding of these adhesions to Fn could result in irreversible attachment to the cell or biofilm production in tissue or bacterial internalization. Expression of Fn-binding proteins is regulated as response to the environmental conditions in which streptococci survive and multiply. Protein F/SfbI, which allows binding to cells of the dermis and Langerhans cells, shows increased expression on bacterial surface with increasing pressure of oxygen. Similarly as in protein F1/SfbI, F2 activity is also response to the environmental oxygen pressure (Jaffe *et al*., 1996). Previous studies showed that the expression of *oprF* and *algD* of *P. aeruginosa* and *Sof* and *Sfbl* of *S. pyogenes* were suppressed after treated with Manuka honey (Maddocks *et al*., 2012; Roberts *et al*., 2012). Honey is a natural product and for many centuries was held in high regard due to its antibacterial properties (Crane, 2001; Rao *et al*., 2016). Such effects have been observed against more than 80 bacterial species, including both Gram-positive and Gram-negative bacteria, and multidrug-resistant pathogens (Cooper *et al*., 2009; Molan, 1992). The inherent antibacterial properties of honey are partly conferred by sugars, which account for 80% of its weight, resulting in a high osmolarity and low water activity (Abu Baker *et al*., 2018; Jibril *et al*., 2019). This study was undertaken to determine the effect of *Trigona* honey on the level of gene expression of *P. aeruginosa* and *S. pyogenes*.

2. Materials And Methods

2.1. Bacterial strains and culture conditions

Pseudomonas aeruginosa ATCC 10145 and *Streptococcus pyogenes* (ATCC 19615) were used throughout the study. One to five colonies of test organism were inoculated into 20 ml nutrient broth (Oxoid, UK) and incubated at 37ºC for 24 hours. After incubation time, the turbidity of the suspension was adjusted to achieve 0.5 McFarland with the absorbance range of 0.08 to 0.1 by using spectrophotometer at wave length of 600 nm (Bouacha *et al*., 2018; Zainol *et al*., 2013).

2.2. Honey Samples

Trigona honey samples were obtained from farm in Kelantan state in East Coast of Peninsula Malaysia. The samples were kept in the dark (Bouacha *et al*., 2018; Garedew *et al*., 2003; Ng *et al*., 2017). The MIC of this *Trigona* honey for the test organisms is 20% (w/v) as described by AL-kafaween *et al*., (2020)

2.3. Growth Kinetics

To determine the effects of *Trigona* honey on the growth of *P. aeruginosa* and *S. pyogenes* cells were grown and treated in 96-well plate with MIC 20% (w/v), half-MIC 10% (w/v) and quarter-MIC 5% (w/v) concentration of honey. Initially, column number 1 was filled 200 µl of final volume of 20%, column number 2 was filled 200 µl of final volume of 10% and column number 3 was filled 200 µl of final volume of 5%. The plate was incubated at 37ºC for 24 hours. At 60 min intervals, the plate was measured at 570nm using a microplate reader (Tecan Infinite 200 PRO, Austria). The experiments were performed in triplicate (Bouacha *et al*., 2018; Maddocks *et al*., 2012; Roberts *et al*., 2012; Zainol *et al*., 2013).

2.4. RNA extraction from P.aeruginosa and S.pyogenes

P. aeruginosa and *S.pyogenes* cells were grown in duplicate in 10 ml of Mueller Hinton broth for 24 hours at 37°C. The total RNA from untreated and treated *P.aeruginosa* and *S.pyogenes* was extracted using the SV Total RNA Isolation System (Promega, UK)(França *et al*., 2011; Goldsworthy, 2008; Maddocks *et al*., 2012; Roberts *et al*., 2012; Wasfi *et al*., 2016; Yadav *et al*., 2012). Total RNA concentrations from untreated and treated of *P.aeruginosa* and *S.pyogenes* were examined by using Implen NanoPhotometer® NP80. RNA purity levels were assessed using the 260/280 absorbance ratio, with only sample ratios between 1.8 and 2.1 being accepted for conversion to cDNA. The experiments were performed in triplicate.

2.5. Conversion of RNA to cDNA

Reverse transcription of RNA was performed with Oligo $(dT)_{15}$ primers and Random Primers. Total RNA samples were converted to cDNA using a high capacity RNA to cDNA conversion kit (Promega, UK). Samples were diluted to 100 ng/µl using ultra pure water. Mastermix 1 was prepared for RNA samples extracted from cells treated and untreated as per the manufacturer's instructions (Promega, UK). For each reaction, 4 µl of (100 ng/µl) RNA, 1 µl of Oligo (dT)15, 2 µl of random primers and nuclease-free water was added to get 10 µl. Mastermix 1 was incubated in PCR thermal cycler at 70°C for 5 minutes to denature the secondary structures of RNA that potentially formed in samples, before was chilled on ice for 5 minutes. While matermix 1 was being incubated, mastermix 2 for each reaction was prepared as following 4 µl 5X Reaction Buffer, 2 µl MgCl2, 1 µl PCR nucleotide mix, 0.5 µl ribonuclease inhibitor, 1 µl reverse transcriptase primers and topped up with nuclease-free water to a final volume of 10 µl. Negative controls were created by substituting total RNA with ultra pure water. The mixture of Mastermix 1 and Mastermix 2 was incubated at 5 minutes at 25ºC, 60 minutes at 42ºC and a final hold at 70ºC for 15 minutes by using a thermal cycler. Following conversion to cDNA, samples were

stored at -20ºC until ready to use (Maddocks et al., 2012; Roberts et al., 2012; Yadav et al., 2012).

2.6. Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR)

Primers of *P. aeruginosa* and *S. pyogenes* were retrieved from previous studies as shown in Table 1. Lyophilised and desalted oligonucleotides were reconstituted using sterile ultra-pure water following the manufacturer's instructions. Stock solutions were stored at -20ºC. The RT-qPCR mastermix for each reaction was prepared by following the manufacturer's instructions (Promega, UK), 10µl of qPCR Master Mix, 1µl of forward primer, 1µl of reverse primer, 2µl of cDNA template, 0.2µl CXR Reference dye and topped up with nuclease-free water to 20µl. Wells were closed with strip caps, centrifuged and placed into PCR instrument. The following PCR protocol was used: denaturation at 95°C for 2 minutes one cycle, amplification at 95°C for 15 seconds 40 cycles and a final elongation annealing: at 60 °C for 1 min 40 cycles. The positive control for the reaction was provided by the manufacturer (Promega), and nano-pure water was used to exclude the possibility of contamination. Also, negative control primers were used for both bacteria. Densitometry was performed by using the Applied Biosystems StepOne Software v2.3. The experiments were performed in triplicate to determine the level of relative gene expression in samples, a modified 2-ΔΔ Ct method was used (Livak and Schmittgen, 2001; Maddocks *et al*., 2012; Roberts *et al*., 2012; Schmittgen and Livak, 2008; Wasfi *et al*., 2016; Yadav *et al*., 2012).

Table 1. Primers used for the RT-qPCR analysis of *P.aeruginosa* and *S.pyogenes*

Gene of name	Amplicon Size (bp)	GC content	Annealing temp (C°)	Number of cycles	Direction	Primer sequence (5' → 3')
1. *algD*	144	42.5 % 42.9%	56	41	Forward Reverse	CGCCGAGATGATCAAGTACA AGGTTGAGCTTGTGGTCCTG
2. *oprF*	101	41.2% 42%	54	41	Forward Reverse	CTGGACGCCATCTACCACTT CTGTCGCTGTTGATGTTGGT
3. *rpoD**	146	42.3% 42.6%	53	41	Forward Reverse	GCGACGGTATTCGAACTTGT CGAAGAAGGAAATGGTCGAG
4. *Sof*	873	36.4 % 42.9 %	57	41	Forward Reverse	ACTTAGAAAGTTATCTGTAGGG TCTCTCGAGCTTTATGGATAG
5. *SfbI*	960	42.9 % 52.6 %	55	41	Forward Reverse	AACTGCTTTAGGAACAGCTTC CCACCATAGCCACAATGCT
6. *glr**	797	42.9 % 47.6 %	54	41	Forward Reverse	ATGGATACAAGACCAATTGG TCATAAGGTGACATGCTCCAC

**rpoD* was used as a reference gene for *P.aeruginosa* and **glr* was used as a reference gene for *S.pyogenes*

2.7. Statistical Analysis

Data was expressed as mean ± standard error of means, one way analysis of variance and graphing was performed using SPSS program, version 20.For each data set, three replicates were performed.

3. Results

3.1. Growth kinetic curves

As shown in Figure 1 and 2, results showed *P.aeruginosa* and *S.pyogenes* could not be recovered after 24 h incubation with MIC (20% (w/v) and prevented the growth of *P.aeruginosa* and *S.pyogenes*. *P. aeruginosa* and *S.pyogenes* treated with half-MIC (10% w/v) have resulted a decreased optical density and decreased lag phase and exponential phase compared to untreated sample. Conversely, *P.aeruginosa* and *S.pyogenes* treated with quarter-MIC concentrations (5 %, w/v) had no inhibition in growth compared with untreated cells. The data obtained from growth kinetics supporting the MIC of *Trigona* honey as mentioned in previous study by Al-kafaween et al., (2020)

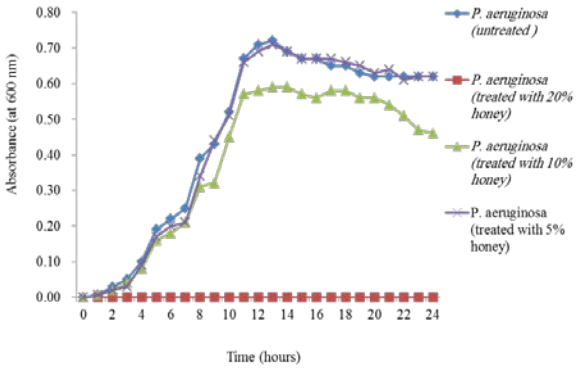

Figure 1. Growth curves of *P.aeruginosa* cells grown with and without *Trigona* honey.

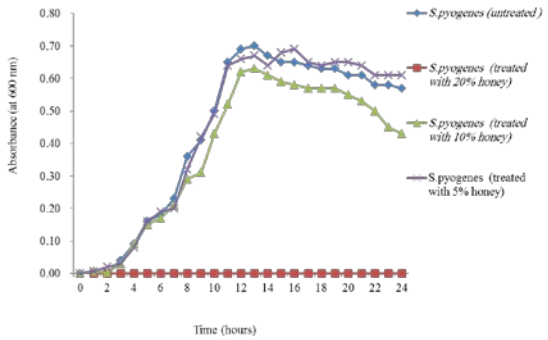

Figure 2. Growth kinetics curve of *S.pyogenes* cells grown with and without *Trigona* honey.

3.2. RT-qPCR of genes expression of P.aeruginosa and S.pyogenes

Two genes *oprF* and *algD* of *P.aeruginosa* and two genes *Sof* and *Sfbl* of *S.pyogenes* showed a statistically significant reduction in gene expression after being treated with 20% (w/v) of *Trigona* honey. As shown in Table 2 and Figure 3, results showed all genes were downregulated and different degrees of downregulation were observed. The RT-qPCR results demonstrated that the expressions of *oprF* and *algD* genes of *P. aeruginosa* were decreased 11.11-fold and 6.28-fold respectively after treated with 20% (MIC) *Trigona* honey. Whereas *Sof* and *Sfbl* of *S. pyogenes* were decreased 7.82-fold and 9.23-fold respectively after treated with 20% (MIC) *Trigona* honey.

Table 2. Effect of *Trigona* honey on the expression of *P.aeruginosa* and *S.pyogenes* detected by RT-qPCR (Schmittgen & Livak, 2008).

Gene name	Average ΔΔCt	Expression Fold Change (2^-ΔΔCt)	Expression Fold Change	*P*-value	SD
1. *oprF*	3.47	0.09	-11.11	0.04*	1.0
2. *algD*	2.65	0.16	-6.28	0.04*	1.5
3. *Sof*	2.97	0.13	-7.82	0.03*	1.3
4. *Sfbl*	3.21	0.11	-9.23	0.03*	1.5

If the delta-delta Ct has a negative value, the gene of interest is upregulated, because the fold change will be larger than 1. On the other hand, if the delta-delta Ct has a positive value, the gene is down regulated and the fold change is <1. *Statistically significant change in the level expression (P<0.05).

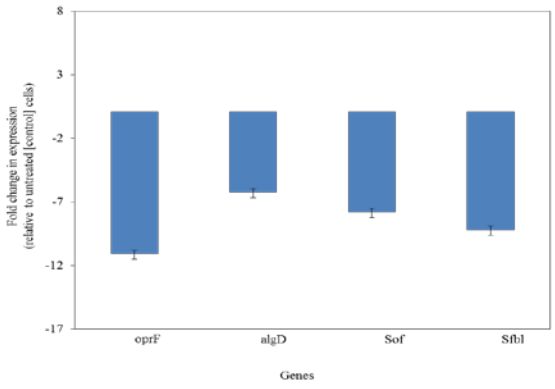

Figure 3. Alterations in gene expression profiles associated with exposure of *P.aeruginosa* and *S.pyogenes* to *Trigona* honey as determined by RT-qPCR. Mean values of fold changes (±SD) are shown in relation to untreated (control).Error bars denote standard error of the mean from three biological samples.

4. Discussion

This study describes the first systematic analysis of the effect of *Trigona* honey on level of gene expression of *P.aeruginosa* and *S. pyogenes*. Total viable cell decreased after exposure to 20% (w/v) and 10% (w/v) of *Trigona* honey. Studies by (Zainol et al., 2013) reported that *Trigona* honey inhibited growth of *P.aeruginosa* at 20% concentration of honey. Studies by (Maddocks et al., 2012; Roberts et al., 2012) showed that the number of cells of *S.pyogenes* were decreased after treated with 20% concentration of Manuka honey. Previous studies showed that the growth kinetics of *P.aeruginosa* and *S.epidermidis* was gradually declined after exposure to 40% (w/v) concentration of Indian honey (Chakraborti *et al*., 2014). Recently, honey has been documented to reduce growth rate of Gram-positive and Gram-negative bacteria (Nassar *et al*., 2011). RT-qPCR was used to determine the level of gene expression of *P.aeruginosa* and *S.pyogenes* after treated with Trigona honey. Reduced expression was noticeable with a different level of expression in both bacteria. The expression of *Sof* and *SfbI* of *S.pyogenes* decreased 7.82-fold and 9.23-fold respectively after treated with 20% (MIC) of *Trigona* honey. Whereas *oprF* and *algD* of *P.aeruginosa* decreased 11.11-fold and 6.28-fold respectively after treated with 20% (MIC) of *Trigona* honey. In previous study *oprF* has been shown to play a role in the anaerobic growth of *P.aeruginosa*(Yoon *et al*., 2002) and subsequent biofilm formation, concurrent with chronic wound infection (Bjarnsholt *et al*., 2008; James *et al*., 2008). The observed reduction of *oprF* in *P.aeruginosa* following treatment with *Trigona* honey may therefore, in part, account for the observed ability of honey-treated *P.aeruginosa* to form microcolonies. GDP-mannose dehydrogenase is essential for the production of *algD* (Leid *et al*., 2005; May *et al*., 1991). It is, therefore, possible that the observed decrease in *algD* and oprF causes instability of the cell envelope, making *P.aeruginosa* susceptible to the osmotic action of *Trigona* honey, which results in the decreased expression of *algD* as the extra cytoplasmic stress response system is activated. Sfbl is regarded as one of the major adhesions of *S.pyogenes* (Medina *et al*., 2000) and Sof is regarded as a major virulence factor that is known to contribute to pathogenesis of streptococcal infection in animal models (Courtney and Pownall, 2010). The *Sof* gene was first sequenced over 15 years ago (Rakonjac *et al*., 1995) and its product was found to be a surface bound protein of over 100 kDa, with a C-terminal domain comprised of numerous repeating peptides that bound to both fibronectin and fibrinogen (Courtney *et al*., 2003; Courtney and Pownall, 2010). It is a possibility that the reduction in fibronectin binding was a combination of reduced expression and specific physical disruption of binding or stearic hindrance by components of the *Trigona* honey.

A study by (Roberts *et al*., 2012) showed that *algD* of *P.aeruginosa* increased 16-fold in the expression whereas *oprF* decreased 10-fold after treated with 12% (MIC) of Manuka honey. A previous study showed that the *Sof* and *Sfbl* proteins decreased in the expression of *S. pyogenes* after treated with 20% (MIC) of Manuka honey (Maddocks *et al*., 2012). A study by (Roberts *et al*., 2014) showed that six genes (*fliA, fliC, flhF, fleN, fleQ* and *fleR*) of *P. aeruginosa* were reduced in gene expression after exposure to 24% (MIC) of Manuka honey. A previous study showed that *tnaA* and *yjfO* (*bsmA*) genes were down-regulated in expression of *E.coli* in the range of 12.5–16.2-fold after treated with 25% (MIC) of Egyptian honey (Wasfi *et al*., 2016). A study by (Wasfi *et al*., 2016) reported that *ycfR* (*BhsA*) and *evgA* genes of *E.coli* were upregulated in expression in the range of 2.2–4.19-fold and 1.09-fold respectively after treated with 25% (MIC) of Egyptian honey. Honey is a complex substance estimated to be comprised of between 200 and 600 components,

including fructose (~38.2 %), glucose (~31.3 %), sucrose (1 %) and 'other sugars' (9 %) (Bogdanov et al., 2008). Additional minor constituents include acids (0.57 %), proteins (0.266 %), amino acids (0.1 %), nitrogen (0.41 %), minerals (0.17 %) The antibacterial action of Trigona honey is attributed to its high osmolarity, low water activity, viscosity, low PH and the presence of hydrogen peroxide; it is the combination of these factors that is thought to provide an unsuitable environment for bacterial growth (Cooper, 2008; Adams et al., 2009). It is evident that Trigona honey is effective at inhibiting the growth of *P. aeruginosa* and *S. pyogenes*, causing abnormal cell by reducing structural integrity to the point of cell lysis as mentioned in previous study by (Al-kafaween et al., 2019). The data presented here supports previous findings and describes the effects of *Trigona* honey on *P. aeruginosa* and *S. pyogenes* at a genetic level. These effects may be compounded by the high osmolarity of *Trigona* honey. Whether these are the only targets remains to be determined, and the global effect of *Trigona* honey on *P. aeruginosa* and *S. pyogenes* will be the subject of future research.

5. Conclusion

This is the first comprehensive study of the level of gene expression of *P. aeruginosa* and *S. pyogenes* after exposure to Malaysian *Trigona* honey. Taken together, our results revealed that the tested Trigona honey has the potential to be effective inhibitors of *S.pyogenes*. Differential gene expression in response to honey exposure exhibited down-regulation of two genes involved in microcolonies and biofilm formation in *P. aeruginosa* and *S. pyogenes.* The obtained results indicate that the honey under study may represent promising antibacterial, antibiofilm and anti-virulence agents for treatment and modulation of infections caused by *P.aeruginosa* and *S.pyogenes*. Future clinical evidence pertaining to the efficacy of the tested *Trigona* honey in the prevention and treatment of *P. aeruginosa* and *S. pyogenes* induced infections at various tissue/cell types might be required.

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