



## In silico SYBR® green-based qPCR primer design for virulent protein C gene of *Edwardsiella tarda*

Rian Ka Praja<sup>1\*</sup>, Reny Rosalina<sup>2</sup>

<sup>1</sup> Biomedical Sciences Program, Faculty of Medicine, Udayana University, Denpasar, Bali, Indonesia, 80113.

<sup>2</sup> Chemistry Department, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jawa Barat, Indonesia, 40132.

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### ABSTRACT

*Edwardsiella tarda* infection in fish results in economic losses in aquaculture industries worldwide. Besides, this pathogen is also a zoonotic agent causing gastrointestinal disease in humans. Detection of virulence factors expressed by *E. tarda* is a key for molecular diagnosis. This study aimed to design SYBR® Green-based quantitative PCR (qPCR) primer for *E. tarda* virulent protein C (EtpC) gene. A sequence of EtpC with accession number AY424360.4:6500-6991 obtained from GenBank NCBI was selected as the basis for qPCR primer design. qPCR primers were designed using Primer3 online software. Further analysis related to the secondary structure of each primer was carried out using Beacon Designer Free Edition. Five pairs of qPCR primers were successfully designed with Primer3. Based on the results of Primer3 and Beacon Designer analysis, F primer pair 1 (5'-GAATCCACCGACGATAAGCACAAA-3') and R primer pair 1 (5'-GACACGCAGCACCGACATCA-3') were the most favorable primer set since this primer met the criteria in terms of length, melting temperature, GC content, and self complementarity. In addition, this primer set had the highest (closest to zero)  $-\Delta G$  value of cross dimer, self dimer, and hairpin. It can be concluded that SYBR® Green-based qPCR primer set for EtpC has been successfully designed. However, laboratory optimization experiments are essential for this designed primer set.

### Introduction

*Edwardsiella tarda* is the causative pathogen of edwardsiellosis in fish and its infection globally results in economic losses in aquaculture (Park *et al.*, 2012). Several other studies successfully identified this pathogen from mammals and birds (Gabriel *et al.*, 2019; Davies *et al.*, 2018; Wang, 2012). In addition, *E. tarda* possesses moderate zoonotic potential in immunocompromised humans and is likely to be an opportunistic pathogen (Kamiyama *et al.*, 2019; Morrisette *et al.*, 2019; Xie *et al.*, 2015). Although it has a wide host range, *E. tarda* has been mainly involved in disease outbreaks in cultured fish and is known as one of the most significant pathogenic bacteria in worldwide aquaculture (Xu and Zhang, 2014).

Edwardsiellosis commonly occurs in aquaculture with imbalanced environmental factors (Kerie *et al.*,

2019). *Edwardsiella tarda*-infected fish exhibit abnormality in swimming, including spiral movement and floating near the surface of the water (Mohanty and Sahoo, 2007). Although *E. tarda* infection has a variety of clinical signs after onset, *E. tarda*-infected fish show opacity of the eyes, exophthalmia, loss of pigmentation, petechial hemorrhage in fin and skin, swelling of the abdominal surface, and rectal hernia. In addition, bloody and watery ascites in the abdominal space and congested kidney, liver, and spleen, are also reported in *E. tarda*-infected fish (Park *et al.*, 2012).

In humans, *E. tarda* is primarily associated with gastrointestinal disease, but the number of reports of extraintestinal disease has increased. A study in Okayama, Japan during January 2005-December 2016 showed *E. tarda* can cause bacteremia (Kamiyama *et al.*, 2019). It has been reported that in

\* Corresponding author.

Email address: [riankapraja@gmail.com](mailto:riankapraja@gmail.com)

Taiwan from 1998 to 2001 that *E. tarda* has been isolated from 22 clinical samples with a variety of extraintestinal manifestations including bacteremia, peritonitis, liver abscess, skin and soft tissue infection, intra-abdominal abscess, biliary tract infection, and tubo-ovarian abscess (Wang *et al.*, 2005). Furthermore, *E. tarda* infections in humans may lead to systemic infection and potentially lethal (Leung *et al.*, 2012).

*Edwardsiella tarda* virulent protein C (*EvpC*) is one of the important virulence factors expressed by *E. tarda*. *EvpC* is part of the type VI secretion system (T6SS). T6SS plays important role in adherence, penetration, survival, and replication of *E. tarda* in epithelial cells and phagocytes (Park *et al.*, 2012). Molecular diagnosis of the infectious agent can be done by detecting the presence of virulence factor genes expressed by the pathogen. One of the commonly used molecular techniques for the aquatic pathogen is polymerase chain reaction (PCR) (Prajna *et al.*, 2019; Sukrama *et al.*, 2017). There are several variations of PCR technique and quantitative real-time PCR (qPCR) has been widely used in the molecular diagnosis of pathogens (Kralik and Ricchi, 2017; Liu *et al.*, 2019; Yoshii *et al.*, 2017). To the best of our knowledge, the use of *EvpC* for detecting the presence of *E. tarda* molecularly is very rare. Thus, this study aimed to design SYBR® Green-based qPCR primer to detect the presence of *EvpC*.

## Materials and Methods

### Edwardsiella tarda virulent protein C gene sequence

Gene sequence of *E. tarda* virulent protein C was retrieved from GenBank NCBI (<https://www.ncbi.nlm.nih.gov>) through the nucleotide search menu. A complete sequence of type VI secretion system gene cluster with accession number AY424360.4 was selected in this study. Furthermore, only the 6500-6991 region encoding *EvpC* was used for the qPCR primer design.

### SYBR® Green-based qPCR Design

Primer3 online software (<http://bioinfo.ut.ee/primer3/>) was used to design SYBR® Green based-qPCR primer. A sequence of *EvpC* AY424360.4: 6500-6991 was employed as the input and the specific parameters associated with the primer design of qPCR referred to Thornton and Basu (2011) with minor modifications (Thornton & Basu, 2011) (Table 1). Other parameters such as “Objective Function Penalty Weights for Primers” and “Objective Function Penalty Weights for Primer

Pairs” followed the same setting as Thornton and Basu (Thornton & Basu, 2011).

### Analysis of primer secondary structure

Beacon Designer Free edition (<http://www.premierbiosoft.com>) was employed for primer secondary structure analysis. In the oligo analysis part, SYBR® Green was selected with a specific parameter as shown in Table 2.

**Table 1.** Parameter used in qPCR primer design by Primer3.

Parameter	Setting
<b>Primer Criteria</b>	
Product size	80-150 and 100-200
Number of return	5
Max 3' stability	9
Max repeat mispriming	12
Pair max repeat mispriming	24
Max template mispriming	12
Pair max template mispriming	24
<b>General Primer Picking Conditions</b>	
Primer size	Min: 20; Opt: 25; Max: 28
Primer Tm	Min: 60; Opt: 64; Max: 70
Max Tm difference	2
Table of thermodynamic parameters	SantaLucia 1998
Product Tm	Opt: 50
Primer GC%	Min: 35; Opt: 65; Max: 80
Max self complementarity	3
Max 3' self complementarity	2
Max #N's	0
Max poly-X	2
Inside target penalty	Default
Outside target penalty	Default
First base index	Default
GC clamp	Default
Concentration of monovalent cations	50
Salt correction formula	SantaLucia 1998
Concentration of divalent cations	3.5
Concentration of dNTPs	0.20
Annealing oligo concentration	Default

**Table 2.** Parameter used in Beacon Designer Software.

Parameter	Setting
Nucleic Acid Concentration	0.25 nM
Mono Ion Concentration	50 mM
Free Mg <sup>++</sup> Concentration	3 mM
Total Na <sup>+</sup> Concentration	269.09 mM

## Results

Based on the results of the nucleotide sequence search on GenBank NCBI, the *EvpC* sequence with

accession number AY424360.4 was selected as the basis for the qPCR primer design. This selected

**Table 3.** Left and right primer statistics.

Primer	Considered	Bad GC%	Tm too low	Tm too high	High any compl	High 3' compl	Poly-X	Fulfilled criteria
Left	1242	452	134	231	213	12	139	61
Right	1113	321	136	227	229	11	141	48

sequence encodes the type VI secretion system gene cluster from *E. tarda*. Then, only region 6500-6991 which specifically encodes *EvpC* was used to design the SYBR® Green-based qPCR primer. Sequence of *EvpC* AY424360.4:6500-6991 retrieved from GenBank NCBI is shown as below:

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evpC_E._tarda      ATG GCT TTT GAT ACT TAT
ATC AAA CTG GAT AAG GTT GAT [ 39]
evpC_E._tarda      GGG GAA TCC ACC GAC GAT
AAG CAC AAA AAA TGG ATT GAA [ 78]
evpC_E._tarda      GTG CTG GGT TTT GCC TGG
GGC GCG GGC AAT GAA TGC ACG [117]
evpC_E._tarda      ATG GAG AGC GGC ACC CAG
GGG CTG AAT ACC GGT AAG GCG [156]
evpC_E._tarda      ATG ATG TCG GTG CTG CGT
GTC ACC AAA TGG ATG GAC TGC [195]
evpC_E._tarda      GCC AGT GTC AAG CTG GCC
TCC GCC GCC GTG CAG GGG CAG [234]
evpC_E._tarda      AAC TTT CCC ACG CTG GAG
CTG GAG ATT TGC ACC CAG GCG [273]
evpC_E._tarda      GGC GAT AAG TTC GCC TTC
TGC ATC TAC AAA TTT ACG CAT [312]
evpC_E._tarda      GTC GCC GTC TCC AGC TAT
CAA TGC TCA GGG GCC ACG GGC [351]
evpC_E._tarda      GGC AGC GAT CGC CCG CAG
GAA ACC ATT GAT TTC GCT TAT [390]
evpC_E._tarda      AAA GAA GTG ACA TGG GAA
TAC GTT CCC CAG GAT CAG AAC [429]
evpC_E._tarda      GGC AAG GCG GGC GGC AAA
ATT GGT CCT GAG GGC TGG AGC [468]
evpC_E._tarda      CTT ATT ACC AAC AAG AAA
AAG TAA [492]

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*EvpC* sequence AY424360.4:6500-6991 was employed for designing SYBR® Green-based qPCR primer with Primer3 software. Primer statistics showed that in total 1242 left and 1113 right primers were considered by Primer3 software. However, 452 left primers and 321 right primers had bad GC%. 347 left primers and 363 right primers did not meet the criteria for Tm. Some others were not favorable related to high 3' complementarity and poly-x (Table 3).

Primer pair statistics showed that 2928 pairs of primers were considered by Primer3 but 2116 of them did not meet the product size criteria, 651 pairs had large Tm difference, and 150 pairs had high complementarity. Only eleven pairs met the criteria

(Table 4). The number of returns we used was five (Table 1) therefore only five pairs of primer were generated by Primer3 even though eleven pairs met the criteria. Five pairs of primers generated by Primer3 showed a variety of length, Tm, GC%, and self 3' complementarity but all primers had three self complementarities (Table 5).

**Table 4.** Primer pair statistics.

Considered	Unacceptable product size	Tm diff too large	High any compl	Fulfilled criteria
2928	2116	651	150	11

## Discussion

The good PCR results are dependent on several factors namely template, Taq polymerase and buffer solutions, and primer (Lorenz, 2012). The primer set used must meet important two parameters, specificity, and efficiency. Specificity is pivotal since mispriming can occur when primers are not well designed (Kusmiyati *et al.*, 2018; Thornton and Basu, 2011). This condition will lead to non-specific amplification. Additionally, efficiency is also critical for PCR reaction as an efficient primer pair will produce a twofold increase in amplicon for each cycle of the PCR (Thornton and Basu, 2011).

In a qPCR application with SYBR® Green as its fluorescence, specificity is very critical. In the reaction mix, SYBR® Green will intercalate to any dsDNA, thus nonspecific amplification products will generate invalid data. Other factors to be considered are the formation of primer dimers and efficiency. Primer dimers elevate fluorescence, resulting in an incorrect quantity of the amplicon. The efficiency (how well the primers perform) of a qPCR reaction should be more than 90–100% (Thornton and Basu, 2011). Efficient primers improve the sensitivity of quantification and allow for assay reproducibility (Quellhorst and Rulli, 2012). Factors affecting the efficiency of a qPCR are the amplicon length and

primer quality. Briefly, the key to favorable SYBR® Green-based primers is to find a pair of primers that are very specific without any primer dimers, relatively generate short amplicons, and are efficient enough to produce consistent results and reproducible.

Understanding common parameters in primer design can help to find favorable primer set (Elsalam, 2003; Thornton and Basu, 2011).

**Table 5.** qPCR primer pairs generated by Primer3 software.

Sequence (5'→3')	Length	Tm	GC%	Self compl.	Self 3' compl.
<b>Primer Pair 1</b>					
F: GAATCCACCGACGATAAGCACAAA	24	64.16	45.83	3	None
R: GACACGCAGCACCGACATCA	20	65.78	60	3	1
Product Length 135 bp					
<b>Primer Pair 2</b>					
F: GAATCCACCGACGATAAGCACAAA	24	64.16	45.83	3	None
R: TGACACGCAGCACCGACATC	20	65.78	60	3	2
Product Length 136 bp					
<b>Primer Pair 3</b>					
F: GATGATGTCGGTGCTGCGTGT	21	66.03	57.14	3	None
R: AACTTATCGCCCGCCTGGGT	20	66.62	60	3	1
Product Length 129 bp					
<b>Primer Pair 4</b>					
F: GATGATGTCGGTGCTGCGTGT	21	66.03	57.14	3	None
R: GAACTTATCGCCCGCCTGGGT	21	67.33	61.90	3	1
Product Length 130 bp					
<b>Primer Pair 5</b>					
F: ATGATGTCGGTGCTGCGTGT	20	65.25	55	3	None
R: AACTTATCGCCCGCCTGGGT	20	66.62	60	3	1
Product Length 128 bp					

**Table 6.** qPCR primer secondary structure analysis using Beacon Designer.

Sequence (5'→3')	GC Clamp	Cross Dimer (ΔG) kcal/mol	Self Dimer (ΔG) kcal/mol	Hairpin (ΔG) kcal/mol
<b>Primer Pair 1</b>				
F: GAATCCACCGACGATAAGCACAAA	1	-0.4	-0.4	-0.4
R: GACACGCAGCACCGACATCA	1	-0.4	0.0	0.0
<b>Primer Pair 2</b>				
F: GAATCCACCGACGATAAGCACAAA	1	-0.4	-0.4	-0.4
R: TGACACGCAGCACCGACATC	1	-0.4	0.0	0.0
<b>Primer Pair 3</b>				
F: GATGATGTCGGTGCTGCGTGT	1	-2.9	0.0	0.0
R: AACTTATCGCCCGCCTGGGT	1	-2.9	-2.0	-2.0
<b>Primer Pair 4</b>				
F: GATGATGTCGGTGCTGCGTGT	1	-2.9	0.0	0.0
R: GAACTTATCGCCCGCCTGGGT	1	-2.9	-2.0	-2.0
<b>Primer Pair 5</b>				
F: ATGATGTCGGTGCTGCGTGT	1	-2.9	0.0	0.0
R: AACTTATCGCCCGCCTGGGT	1	-2.9	-2.0	-2.0

The primer design is the first step that determines the performance of DNA amplification using the PCR method (Praja, 2021). Things considered in the selection of a primer set include the length of the primer, melting temperature (Tm), GC%, and free energy (ΔG) of cross dimer, self dimer, and hairpin (Pradnyaniti et al., 2013). The length of oligonucleotide primer ranges from 18-24 nt. Longer primers will take a longer time to hybridize, longer to extend, and longer to remove thus produces less

amplicon. The second characteristic to be considered in primer selection is Tm. A favorable primer set has a Tm difference of around 5°C. The percentage between bases G and C also needs to be considered because the content of the number of bases G and C is related to Tm of a primer. Ideally, a primer has a percentage of G and C around 40-60% (Pradnyaniti et al., 2013; Saraswati et al., 2019). Other criteria for ideal primers are having low number with the highest

-ΔG (closest to zero) of cross dimer, self dimer, and hairpin (Thornton and Basu, 2011).

Analysis of forward primer pair 1 (5'-GAATCCACCGACGATAAGCACAAA-3') and reverse primer pair 1 (5'-GACACGCAGCACCGACATCA-3') showed a product length of 135 bp which was in position 43-177 of *EvpC* gene (Figure 1). Analysis results showed that primer pair 1 was the most ideal primer pair to amplify *EvpC* gene among others since this primer set met the criteria for the length of nucleotides, small Tm temperature difference (1.62°C), GC%, low self 3' complementarity, and had the highest -ΔG of cross dimer, self dimer, and hairpin.



**Figure 1.** Illustration of region amplified by primer pair 1 marked by >>> and <<<. Forward and reverse primer pair 1 start between region 43 and 177, respectively with a product size of 135 bp.

**Conclusions**

A SYBR® Green-based qPCR primer set to detect *EvpC* has been successfully designed. Based on this in silico study, primer pair 1 generated by Primer3 software is the most efficient primer set among others. Primer pair 1 meets the criteria as an ideal qPCR primer. However, laboratory experiments are needed to validate and optimize this primer set.

**References**

Davies, Y.M., M.G.X. de Oliveira, M.P.V. Cunha, L.S. Franco, S.L.P Santos, L.Z. Moreno, V.T. de Moura Gomes, M.I.Z. Sato, M.S. Nardi, A.M. Moreno, A.B. Saldenberg, L.R.M. de Sa, T. Knöbl. 2018. *Edwardsiella tarda* outbreak affecting fishes and aquatic birds in Brazil. *Veterinary Quarterly*, 38(1), 99–105. <https://doi.org/10.1080/01652176.2018.1540070>

Elsalam, K.A.A. 2003. Bioinformatic tools and guideline for PCR primer design. *African Journal of Biotechnology*, 2(5), 91–95.

Gabriel, M.E., L.C. Henker, C. Carneiro, A. Gris, R.A. Casagrande, T.M. Augusto Gomes, D.M. dos Santos, R.E. Gomes. 2019. Typhlocolitis by *Edwardsiella tarda* in a Cow. *Acta Scientiae Veterinariae*, 47(1), 381. <https://doi.org/10.22456/1679-9216.91420>

Kamiyama, S., A. Kuriyama, T. Hashimoto. 2019. *Edwardsiella tarda* Bacteremia, Okayama, Japan, 2005–2016. *Emerging Infectious Diseases*, 25(10), 1817–1823. <https://doi.org/https://doi.org/10.3201/eid2510.180518>

Kerie, Y., A. Nuru, T. Abayneh. 2019. *Edwardsiella* Species Infection in Fish Population and Its Status in Ethiopia. *Fisheries and Aquaculture Journal*, 10(2). <https://doi.org/10.35248/2150-3508.19.10.266>

Kralik, P., M. Ricchi. 2017. A basic guide to real time PCR in microbial diagnostics: Definitions, parameters, and everything. *Frontiers in Microbiology*, Vol. 8, p. 108. *Frontiers Research Foundation*. <https://doi.org/10.3389/fmicb.2017.00108>

Kusmiyati, F., S. Sutarno, B. Hervibawa. 2018. In Silico Design of PCR Primers to Amplify the Salt Tolerance Gene in Soybean. *Asian Journal of Applied Sciences*, 6(6), 509–514. <https://doi.org/10.24203/ajias.v6i6.5532>

Leung, K. Y., B.A. Siame, B.J. Tenkink, R.J. Noort, Y.K. Mok. 2012. *Edwardsiella tarda* – Virulence mechanisms of an emerging gastroenteritis pathogen. *Microbes and Infection*, 14(1), 26–34. <https://doi.org/10.1016/j.micinf.2011.08.005>

Liu, Y., Y. Cao, T. Wang, Q. Dong, J. Li, C. Niu. 2019. Detection of 12 Common Food-Borne Bacterial Pathogens by TaqMan Real-Time PCR Using a Single Set of Reaction Conditions. *Frontiers in Microbiology*, 10(FEB), 222. <https://doi.org/10.3389/fmicb.2019.00222>

Lorenz, T.C. 2012. Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments*, 63(63), 3998. <https://doi.org/10.3791/3998>

Mohanty, B.R., P.K. Sahoo. 2007. *Edwardsiellosis* in fish: A brief review. *Journal of Biosciences*, Vol. 32, pp. 1331–1344. *Indian Academy of Sciences*. <https://doi.org/10.1007/s12038-007-0143-8>

Morrisette, T., W.P. Hewgley, H. Hewgley. 2019. *Edwardsiella tarda* Bacteremia in Untreated Hepatitis C: Alterations in Antimicrobial Therapy for a Pan-Susceptible Pathogen in a Critically Ill Patient. *American Journal of Therapeutics*, 26(4), e530–e533. <https://doi.org/10.1097/MJT.0000000000000958>

Park, S.B., T. Aoki, T.S. Jung. 2012. Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. *Veterinary Research*, 43(1), 1. <https://doi.org/10.1186/1297-9716-43-67>

Pradnyaniti, D.G., IN. Wirajana, S.C. Yowani. 2013. Desain Primer secara in silico untuk Amplifikasi *Fragment Gen rpoB Mycobacterium tuberculosis* dengan Polymerase Chain Reaction (PCR). *Jurnal Farmasi Udayana*, 124–130.

Praja, R.K. 2021. In silico oligonucleotide primer design for *Campylobacter jejuni* cytolethal distending toxin B gene amplification. *Oceana Biomedicina Journal*, 4(1), 53–65. <https://doi.org/10.30649/OBJ.V4I1.88>

Praja, R.K., ID.M. Sukrama, N.N.D. Fatmawati. 2019. Detection of genes encoding ompW and ctxA of *Vibrio cholerae* isolated from shrimp and shellfish at Kedonganan fish market, Bali-Indonesia. *Oceana Biomedicina Journal*, 2(1), 1–14.

Quellhorst, G., S. Rulli. 2012. A systematic guideline for developing the best real-time PCR primers Lessons learned from designing assays for more than 14, 000 genes. *Qiagen*, 1–9.

Saraswati, H., F.D. Wahyuni, S. Seprianto. 2019. Desain Primer Secara In Silico untuk Amplifikasi Gen *cryIII* dari *Bacillus thuringiensis* Isolat Lokal. *Indonesian Journal of Biotechnology and Biodiversity*, 3(1), 33–38.

Sukrama, ID.M., R.K. Praja, N.N.D. Fatmawati. 2017. Pheno-genotypic profile of *Vibrio cholerae* hemolysin (*hlyA*) isolated from shrimp and shellfish at the Kedonganan fish market, Bali-Indonesia. *Bali Medical Journal*, 5(2), 366–369. <https://doi.org/10.15562/bmj.v5i2.231>

Thornton, B., C. Basu. 2011. Real-time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education*, 39(2), 145–154. <https://doi.org/10.1002/bmb.20461>

Wang, I.K., H.L. Kuo, Y.M. Chen, C.L. Lin, H.Y. Chang, F.R. Chuang, M.H. Lee. 2005. Extraintestinal manifestations of *Edwardsiella tarda* infection. *International Journal of Clinical Practice*, Vol. 59, pp. 917–921. *Int J Clin Pract*. <https://doi.org/10.1111/j.1742-1241.2005.00527.x>

Wang, X. 2012. Identification of *Edwardsiella tarda* isolated from duck and virulence genes detection. *African Journal of Microbiology Research*, 6(23). <https://doi.org/10.5897/ajmr11.1604>

Xie, H.X., J.F. Lu, Y. Zhou, J. Yi, X.J. Yu, K.Y. Leung, P. Nie. 2015. Identification and functional characterization of the novel *Edwardsiella tarda* effector EseJ. *Infection and Immunity*, 83(4), 1650–1660. <https://doi.org/10.1128/IAI.02566-14>

Xu, T., X.H. Zhang. 2014. *Edwardsiella tarda*: An intriguing problem in aquaculture. *Aquaculture*, Vol. 431, pp. 129–135. Elsevier. <https://doi.org/10.1016/j.aquaculture.2013.12.001>

Yoshii, Y., K. Shimizu, M. Morozumi, N. Chiba, K. Ubukata, H. Uruga, S. Hanada, H. Wakui, S. Minagawa, H. Hara, T. Numata, K. Saito, J. Araya, K. Nakayama, K. Kishi, K. Kuwano. 2017. Detection of pathogens by real-time PCR in adult patients with acute exacerbation of bronchial asthma. *BMC Pulmonary Medicine*, 17(1), 150. <https://doi.org/10.1186/s12890-017-0494-3>