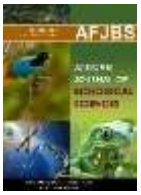
https://doi.org/10.48047/AFJBS.6.13.2024. 2632-2644

African Journal of Biological Sciences



Detection of InvA Gene and Antibiotic Susceptibility Pattern of Indigenous *Salmonella typhi* Isolates

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Article History
Volume 6, Issue 13, 2024
Received: 18June 2024
Accepted: 02July 2024
doi:*10.48047/AFJBS.6.13.2024.* 2632-2644

ABSTRACT

The invA gene is a virulence factor in *Salmonella typhi* bacteria that can cause the bacteria to invade intestinal epithelial cells and produce toxic compounds that are pathogenic. The invA gene is also known to be resistant to certain antibiotics. This study was conducted to determine the profile of antibiotic resistance from collection of *S. typhi* isolates that were detected to have the invA gene. Antibiotic resistance was tested using the Kirby-Bauer method. Meanwhile, the invA gene (284 bp) was detected using the primers IAF (5' GTGAAATTATCGCCACGTTCGGGCAA 3') and IAR (5'TCATCGCACCGTCAAAGGACCC 3'). The antibiotic resistance of S. typhi isolates to the ten types of antibiotics was very diverse. Two of the 14 isolates tested, namely *S. typhi* BPE 121.1MC and BPE 122.4CCA were known to have the highest Multiple Antimicrobial Resistance Index. Imipenem and piperacillin were the most effective antibiotics with an inhibition zone of ≥22 mm. Meanwhile, ceftazidime is classified as the least effective antibiotic for inhibiting *S. typhi*. Most of the isolates tested were multidrug resistant. The invA gene was detected in all tested isolates. Based on the results, caution is needed in the use of antibiotics because they have begun to show a decrease in effectiveness.

Key words: antibiotic resistance, invA gene, *Salmonella typhi*.

INTRODUCTION

Salmonella enterica serovar Typhi (*Salmonella typhi*) is a gram-negative bacterium and has special characteristics, namely shaped bacilli, moves using flagella, and does not have spores (Keyser et al., 2018). This bacterium is known to be responsible for several cases of foodborne illness worldwide, is pathogenic, and is involved in the spread of antimicrobial resistance because it can accumulate antibiotic resistance genes (Gargano, 2021). Epidemiological data on the spread of *S. typhi* infection in various food products throughout Indonesia has reached 46.6%. It is recorded that cases of salmonellosis in the world annually reach 93.8 million cases. This figure is not a small number so its existence can be categorized as a high-risk threat that can threaten public health (Zelpina, 2020).

The invA gene is a specific marker gene found in *S. typhi* bacteria. This gene has a feature that makes it as a target gene for the identification of *S. typhi*, namely a low mutation rate. The invA gene in *S. typhi* bacteria is located in an area called Salmonella Pathogenicity Island (SPI). SPI is an area that has many invasive gene clusters that make the bacteria pathogenic (Marcus et al., 2020). SPI in *S. typhi* is divided into 5 types, namely SPI 1-5 with differences in the mechanism of action of each. For the invA gene itself, it is located on SPI-1 which has a mechanism to invade epithelial cells so that it will cause an inflammatory reaction in the intestine (Lou et al., 2019). Therefore, this gene can be used to determine whether the bacterium is pathogenic or not. The invA gene generally has a molecular size of 284 base pairs (Yanestria et al., 2019). The location of invA in *Salmonella* bacteria is on a protein-producing chromosome that can provide invasive properties to invade epithelial cells in the intestine (Muhsinin et al., 2018). Karmi (2013) proved that *Salmonella* mutants that are missing the invA gene are less invasive compared to *Salmonella* that have this set of genes. Therefore, it can be concluded that the *invA* gene is an important virulence factor for *S. typhi* (Liwan and Budiarso, 2018).

Antibiotics are one way of treatment to fight life-threatening infections. However, the misuse and overuse of existing antimicrobials in humans, animals, and plants have accelerated the development and spread of Antimicrobial Resistance (AMR) (Siddiky, 2021). Excessive and inappropriate use of antibiotics is the main factor associated with the increase in antibiotic-

resistant bacteria. Antibiotic-resistant bacteria will survive and continue to reproduce through several mechanisms that allow them to survive during antibiotic treatment (Najwa, 2015). The invA gene along with several other genes located on the Salmonella Pathogenicity Island (SPI) are also thought to provide antibiotic resistance properties such as streptomycin, lincosamide, and chloramphenicol (Karmi, 2013). In this study, an antibiotic resistance test will be carried out on a collection of indigenous *S. typhi* isolates that have been identified in previous studies (Amarantini et al., 2011). Six classes of antibiotics namely penicillin, cephalosporin, carbapenem, aminoglycoside, tetracycline, and quinolone were used in this study. Penicillins, cephalosporins, and carbapenems represent a group of antibiotics that inhibit cell wall synthesis. Aminoglycosides and tetracyclines represent a group of antibiotics that inhibit protein synthesis. While quinolones were chosen to represent groups that inhibit nucleic acid synthesis. In addition to the sensitivity test, it is also necessary to detect the presence of the invA gene because this isolate has not been confirmed for its pathogenic properties which can be seen from the presence or absence of virulence factors in this isolate.

MATERIALS AND METHODS

Salmonella typhi culture preparation

Salmonella typhi isolates were obtained from previous research collections stored in glycerol stocks (Amarantini et al., 2011). As much as 1 ml of bacterial suspension was grown in 3 ml of Brain Heart Infusion (BHI) broth medium and incubated for 24-48 hours until turbidity was seen in the BHI broth medium. Furthermore, the culture was transferred to a petri dish containing BHI medium to obtain a single colony using the streak plate method. Confirmation of the purity of *S. typhi* culture was carried out by carrying out gram staining. The isolates were then stored in BHI medium so that they were slanted for use in antibiotic resistance tests and DNA isolation.

InvA gene detection

The process of detecting the *invA* gene includes DNA isolation, polymerase chain reaction (PCR), and electrophoresis. Bacterial DNA was isolated following the Presto Mini gDNA Bacteria Kit procedure by Geneaid (2017). The invA gene was detected using IAF markers with a base sequence of 5' GTG-AAA-TTA-TCG-CCA-CGT-TCG-GGC-AA 3' and IAR 5' TCA-TCG-CAC-CGT-CAA-AGG-ACC-C 3' (284 bp) following the procedure of Chaudhary et al.,

(2015). The number of PCR cycles used was 35 cycles with PCR conditions namely pre-denaturation 95°C for 5 minutes, denaturation 94°C for 30 seconds, annealing 62°C for 1 minute, extension 72°C for 30 seconds and final extension 72 °C for 10 minutes (Yanestria et al., 2019).

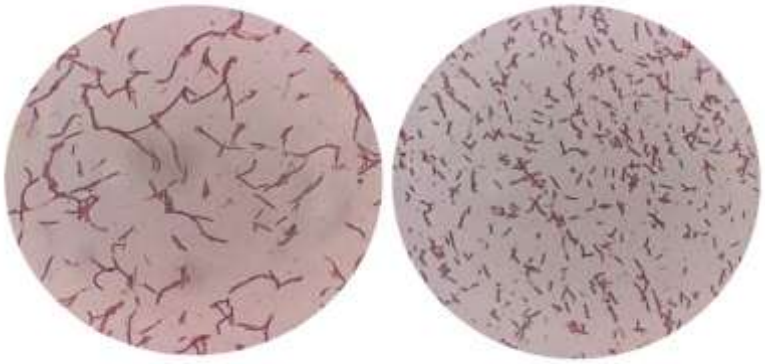
Antibiotic Resistance Test of *S. typhi* Isolates with Disc Diffusion Method

Antibiotic susceptibility test was performed using Mueller Hinton Agar (MHA) medium (Oxoid) according to the procedure of the disc diffusion method (Kirby-Bauer method). *S. typhi* isolates were first grown in liquid BHI broth medium for 18 hours, then grown onto the surface of a petri dish containing Mueller Hinton Agar (MHA) medium by smearing it evenly using a cotton swab. The antibiotic discs used were ampicillin (AMP, 20 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), imipenem (IPM, 10 µg), meropenem (MEM, 30 µg), piperacillin (PRL, 110 µg), gentamicin (CN, 10 µg), tetracycline (TE, 30 µg), amikacin (AK, 30 µg), and ciprofloxacin (CIP, 5µg) (Oxoid). After incubation for 18 hours at 37°C, the inhibition zone was observed. The average value of the diameter of the inhibition zone (mm) measured diagonally, vertically, and horizontally was categorized based on the standards issued by the Clinical Laboratory Standards Institute (CLSI), namely sensitive for sizes ≤14 mm, intermediate for sizes 15-19 mm, and also resistant for the inhibition zone with a size of ≥20 mm (Weinstein, 2021).

RESULTS AND DISCUSSION

Examination of *S. typhi* Culture Purity

Examination of culture purity using the gram staining method is shown in Figure 1. *Salmonella typhi* isolates are gram-negative bacteria so that the cell morphology will appear pink through gram staining (Hamidah et al., 2019; Nurhidayati et al., 2015). Based on the results of gram staining that had been carried out on 14 isolates, all isolates had the same morphological appearance, namely pink in color and in the form of bacilli. In accordance with research conducted by Kasim in 2020, the *S. typhi* bacteria has the characteristics of being in the form of bacilli and is a gram-negative bacterium. These results indicate that the re-cultured *S. typhi* is pure and can be used to test for antibiotic resistance.



(a) (b)

Figure 1. Morphological appearance of *Salmonella typhi* cells as a result of gram staining. Description: (a) BPE 120.1 MC, (b) BPE 121.1 MC

***InvA* Gene Detection**

At this research stage, a series of processes were carried out to detect the presence/absence of the presence/absence of the invasive invA gene in *S. typhi* isolates that had been tested for resistance. Testing for the presence of invasive genes is intended because this gene is the most important virulence factor to determine whether the bacterial isolate is pathogenic or not (Yanestria et al., 2019). IAF primer (5' GTG-AAA-TTA-TCG-CCA-CGT-TCG-GGC-AA 3') and IAR primer (5'TCA-TCG-CAC-CGT-CAA-AGG-ACC-C 3') were used to identify the presence of the invA gene in *S. typhi*. The use of this primer is based on the high specificity of the invA (target) gene for the DNA strand so that this primer will specifically amplify the SPI-1 region where this gene is located in *S. typhi* (Li and Chen, 2013). The invA gene is highly conserved, which means that the gene will not undergo mutations during the evolution process. Therefore, the use of IAF and IAR primers will not detect the presence of other genes in the SPI-1 region (Mohammed et al., 2022).

Figure 2 shows the amplicon of the invA gene (284 bp) as a result of PCR. According to research conducted by Rahn (1992) as the first study on the existence of the invA gene in *S. typhi*, the base size of the DNA fragment of the invA gene owned by *S. typhi* is 284 base pairs. This was confirmed by the results that could be read in electrophoresis by the presence of DNA bands in the 284 base pair column in all tested isolates. The presence of a 284 bp amplicon

illustrates that all isolates have the invA gene. Therefore, it can be believed that all *S. typhi* isolates analyzed have the invA gene as one of the markers of this bacterium that is pathogenic in the human body (Yulian, et al., 2020). The presence of the invA gene in all *S. typhi* isolates indicates that this gene can be used as a biomarker to detect a type of bacteria found in blood samples, food, and so on. This result is reinforced by research that was previously conducted by Mohammed (2022).

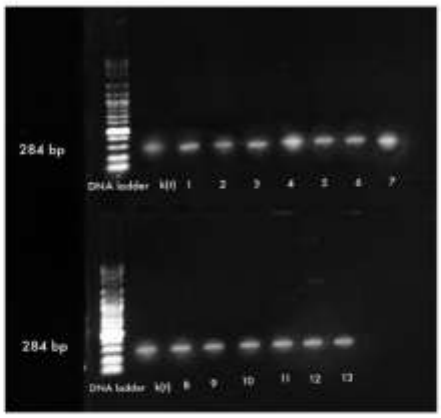


Figure 2. Amplicons of the invA gene detected in *Salmonella typhi* isolates.

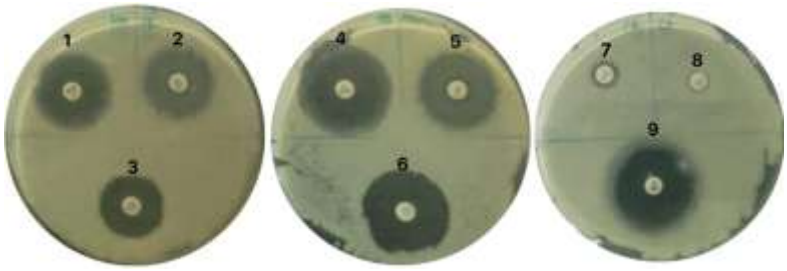
Description: (K (+)) Positive control *S. typhi* NCTC 786, (1) BPE 74.1 CCA, (2) RSK 22.4 CCA, (3) RSK 32.1 SSA, (4) RSL 3.1 SSA, (5) BPE 120.1 MC, (6) BPE 127.1 MC, (7) BPE 122.4 CCA, (8) BPE 123.1 CCA, (9) RSK 5.1 SSA, (10) BPE 121.1 MC, (11) BPE 7.10 MC, (12) RSK 22.2 CCA, (13) BPE 127.2 MC

Resistance Profile of *S. typhi* to Antibiotics

The results of the antibiotic resistance test are known based on the size of the inhibition zone formed. Figure 3 shows the inhibition zones formed for representative test isolates of *S. typhi* strain BPE 122.4 CCA and *S. typhi* strain RSK 22.2 CCA. According to CLSI 2021 (Weinstein, 2021), the standard characteristics of *S. typhi* resistance to antibiotics are divided into several categories, namely: sensitive for inhibition zone sizes >19mm, intermediate for inhibition zone sizes of 15-19mm, and resistant for inhibition zone sizes <14mm. The results of the inhibition zone diameter measurements are tabulated in Table 1 and classified according to the CLSI standard.

Table 1 shows that piperacillin and imipenem were most effective in inhibiting the growth of *S. typhi* with an inhibition zone of ≥ 22 mm. All isolates tested using these two antibiotics have a sensitive resistance profile. Both of these classes of antibiotics are classified as

antibiotics with a mechanism of inhibiting the synthesis of the cell wall. The mechanism of action of antibiotics about inhibiting the formation of the cell wall is by binding to the amino acid D-alanyl-D-alanine as a structural component of the cell wall so that the peptidoglycan-forming layers, namely N-acetylmuramate and N-acetylglucosamine cannot be bound (Ghooi and Thatte 1995). Based on these findings, *S. typhi* is sensitive to piperacillin and imipenem.



(a) (b) (c)

Figure 3. Inhibition zone of *Salmonella typhi* against antibiotics.

Isolates description: (a) BPE 122.4 CCA, (b) RSK 22.2 CCA, (c) BPE 122.4 CCA
Antibiotics description: (1) Meropenem, (2) Tetracycline, (3) Piperacillin, (4) Meropenem, (5) Tetracycline, (6) Piperacillin, (7) Cefotaxime, (8) Ceftazidime, (9) Imipenem

Table 1. Resistance Test Results of *Salmonella typhi* Isolates to Antibiotics

Isolates	Types of Antibiotics										MAR Index
	Cell Wall						Protein			Nucleic Acid	
	AMP	PRL	IPM	MEM	CTX	CAZ	CN	AK	TE	CIP	
RSL 3.1 SSA	2,7	26	28,3	29,3	3	0	18,3	20,3	21,7	23,3	0,3
RSK 5.1 SSA	29	34	30,3	23	28	20	9,3	21,3	23,7	19,3	0,1
RSK 32.1 CCA	11	26,3	30	29	9,3	0	14	14,7	26,7	27,7	0,4
RSK 22.2 CCA	19,3	28	39,3	32,3	8,7	10,7	17,3	25,3	26,7	19	0,2
RSK 22.4 CCA	32	24,3	31,7	27,3	0	0	13,3	9	29,3	23,7	0,4
BPE 7.10 MC	11,7	26	35,3	31,7	11	8,3	21	20	25	26,7	0,3
BPE 120.1 MC	27,3	38	35,7	28,7	30	18	16,3	12,7	28,6	12,7	0,2
BPE 121.1 MC	13	23	28,7	22	6,3	0	12	13,3	24	25,6	0,5
BPE 127.1 MC	34,7	30,7	33,3	25	25,3	22,7	13,3	11,3	11	24,3	0,3
BPE 127.2 MC	29,3	35,3	34,3	28,7	36,3	24,3	10,7	10,7	30	20,3	0,2
BPE 74.1 CCA	23,7	35,3	28,3	25,7	17,7	0	17	10	29	22	0,2
BPE 122.4 CCA	12,7	21,3	28,7	24,7	10	6,7	14	8,7	25,7	25	0,5
BPE 123.1 CCA	12	22	31,7	25,7	15	9,7	15,7	18,7	22,7	31	0,2
S. typhi NCTC 786	34,7	26	31,7	19,3	33,7	23	11,3	25,3	35	23	0,1

Description: White = sensitive, inhibition zone ≥20mm; Grey = intermediate, inhibition zone 14-19mm; Black = resistant, inhibition zone ≤14mm. AMP = Ampicillin, PRL= Piperacillin, IPM = Imipenem, MEM = Meropenem, CTX = Cefotaxime, CAZ = Ceftazidime, CN = Gentamicin, AK = Amikacin, TE = Tetracycline, CIP = Ciprofloxacin.

Furthermore, still, in the cell wall inhibition category, it is also known that meropenem is also very effective in inhibiting *S. typhi* tested isolates, except for *S. typhi* isolate NCTC 786. Table 1 also shows that three of the six antibiotics in the cell wall inhibitor category, namely ceftazidime, cefotaxime, and ampicillin were classified as less effective in inhibiting all tested isolates. Ceftazidime was only able to inhibit the growth of four tested isolates out of a total of 14 tested isolates while cefotaxime was only able to inhibit the growth of five tested isolates out of a total of 14 tested isolates. Only six of the total 14 isolates tested were sensitive to ampicillin. Most of the tested *S. typhi* isolates showed resistance to ceftazidime, cefotaxime, and ampicillin. A similar finding was that resistance to these antibiotics was also found in *S. typhi* isolates tested at the Microbiology Laboratory at Abdullahi Waste Specialist, Kano. It was further stated that resistance to these antibiotics was significantly high in studies conducted in most parts of the world. Plasmids are also known to mediate multi-drug resistance to ampicillin, chloramphenicol, and so on in various parts of Asia (Farouk Nas et al., 2018).

The next category of antibiotics tested was protein synthesis inhibitors. There are 3 antibiotics used namely gentamicin, amikacin, and tetracycline. According to the results of the study, gentamicin and amikacin were considered not effective in inhibiting the growth of *S. typhi* bacteria because they only succeeded in inhibiting 7 isolates for gentamicin and 8 isolates for amikacin from a total of 14 tested isolates. These results are supported by previous research conducted by Mandal et al., (2009) which said that amikacin and gentamicin were not effective in inhibiting the growth of *S. typhi*. This is due to the difficulty of aminoglycoside class antibiotics to penetrate intracellular pathogenic bacterial cell tissues. Therefore, the bacteria remain active and are infectious to the host (Mandal et al., 2009). In contrast to the other 2 classes of antibiotics, tetracycline has test results that are very effective in inhibiting the growth of *S. typhi*. Isolate with code BPE 127.1 MC is the only isolate that has a profile that is resistant to this antibiotic. Based on these results, it can be seen that tetracycline succeeded in inhibiting the bacterial protein synthesis of *S. typhi*. The mechanism of tetracycline in inhibiting protein synthesis is by interacting with the bacterial 30S ribosomal subunit so that it will prevent binding by tRNA in amino acids. Failure of the tRNA to bind will result in inhibition of the codon and

anticodon, which will fail the translation process, resulting in the unavailability of energy for the bacteria to carry out protein synthesis (Anggita et al., 2022).

Ciprofloxacin is a quinolone class of antibiotics with a mechanism of inhibiting nucleic acid synthesis in bacteria. Ciprofloxacin succeeded in inhibiting the growth of 11 isolates out of a total of 14 *S. typhi* isolates tested with a note that 2 isolates had an intermediate profile. The effectiveness of this antibiotic in inhibiting *S. typhi* is supported by research conducted by Girgis (1999) with samples of *S. typhi* from human blood. This study said that the antibiotic ciprofloxacin was effective in inhibiting the growth of *S. typhi*, however, it was necessary to innovate in its use because the use of this antibiotic to inhibit *S. typhi* had begun to show a decrease in effectiveness due to the absence of innovation from year to year (Mutai et al., 2018). Antibiotics class of inhibitors of nucleic acid synthesis have a mechanism of action by intervening in DNA replication. Antibiotics can intervene by binding to the topoisomerase 2 enzyme as an essential enzyme that regulates DNA replication. The topoisomerase 2 enzyme that binds to antibiotics will damage this enzyme so that the DNA in bacteria cannot replicate (Bhattacharjee, 2022).

Table 1 also shows MAR index data. MARI stands for Multiple Antimicrobial Resistance Index. This index is an effective and valid indicator to determine the resistance properties of an isolate to several types of antibiotics tested. The way to get the MARI value is by doing a comparison between the resistant antibiotics and all the antibiotics tested against the same isolate. The higher the MARI value, the more difficult it is to inhibit the growth of the isolate and vice versa. The highest MARI value was owned by isolates BPE 121.1 MC and BPE 122.4 CCA with a value of 0.5 which means that these isolates have high resistance to various types of antibiotics. While the smallest MARI value was owned by 2 isolates, namely RSK 5.1 SSA and the positive control *S. typhi* NCTC 786 with a MARI value of 0.1. This indicates that these isolates are more easily inhibited by various types of antibiotics (Ayandele et al., 2020).

Overall, the results of the antibiotic resistance test profile can be described as shown in Figure 4. The most effective antibiotics to inhibit *S. typhi* were piperacillin and imipenem in the category of inhibiting cell wall synthesis. The results of the resistance test also showed that not all of the cell wall-blocking antibiotics were effective against *S. typhi*. The least effective cell wall inhibitor category antibiotic against *S. typhi* was ceftazidime with the ability to only inhibit 3 out of 14 tested isolates.

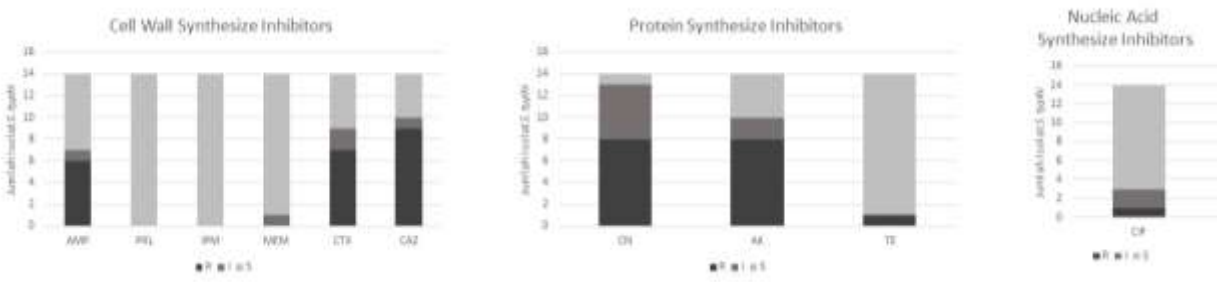


Figure 4. *Salmonella typhi* resistance profile to antibiotics.

Description: Ampicillin (AMP), Piperacillin (PRL), Imipenem (IPM), Meropenem (MEM), Cefotaxime (CTX), Ceftazidime (CAZ), Gentamicin (CN), Amikacin (AK), Tetracycline (TE), Ciprofloxacin (CIP)

CONCLUSION

The invA gene with a size of 284 base pairs was detected in each tested *S. typhi* isolates. The antibiotic resistance profile of *S. typhi* isolates to the ten types of antibiotics was very diverse. *Salmonella typhi* isolates were very sensitive to imipenem and piperacillin. Meanwhile, ceftazidime is classified as the least effective antibiotic in inhibiting *S. typhi*. *Salmonella typhi* isolates NCTC 786 and RSK 5.1 SSA were the strains that were most easily inhibited by various antibiotics, while isolates BPE 121.1 MC and BPE 122.4 were the bacteria that were most difficult to inhibit its growth.

ACKNOWLEDGEMENT

This research was funded by the Faculty of Biotechnology, Universitas Kristen Duta Wacana in 2023 with contract number: 123/D.01/Bio/2023. The researcher is grateful for the financial support that has been given.

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