American Journal of BioMedicine
2017; **5**(1): 16-31

Immunogenic properties of outer membrane protein of *Acinetobacter baumannii* that loaded on chitosan nanoparticles

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Abstract

This study aimed to prepare an experimental nano vaccine for *Acinetobacter baumannii*. In current study, Acinetobacter isolates were identified by growth onto CHRO Magar TM Acinetobacter/MDR and standard biochemical tests. Identification of isolates of Acinetobacter isolates to species level was done by VITEK 2 compact system and API20. Chitosan nanoparticles was used in this study as an adjuvant, the results showed chitosan a perfect adjuvant because it carried outer membrane protein antigen and stimulates an immune response at treated rats, use of Chitosan nanoparticles as a carrier to OMP increased the concentration of cytokines (IL-2, IL-6, IFN-ɣ), antibody titer, total leukocytes and differential leukocytes in treated rats compared with each control group and OMP group.

Keywords: Vaccine; IL-2; IL-6; INFɣ; WBCs count

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Received 11 August 2014; accepted 25 December 2014; published 29 January 2015

Introduction

Acinetobacter is gram-negative, non-motile, strictly aerobic bacteria; it is a heterogeneous group of organisms that is ubiquitous, widely distributed in nature [1]. This bacterium is well adapted to survive and tolerates both wet and dry hospital environment conditions [2]. Moreover, sources of *Acinetobacter* transmission was identified in the outbreaks in health care setting include medical devices and equipment's such as ventilator circuits, spirometers, suction catheters, etc [3-5].

The challenges of treating multidrug-resistant bacteria continue to be at the front of the clinician's practice in caring for hospitalized patients [6]. Progress in the development

of vaccines and vaccination procedures have helped to prevent and in some cases eliminate diseases in humans, farm and companion animals [7]. One of the important factors contributing to this drug resistance is perhaps the impermeability of certain classes of antibiotics across the Outer membrane (OM) or some structural change in the proteins of the (OM) [8].

Vaccines utilising pure antigens instead of whole pathogens and alternative administration routes require the use of potent adjuvants and effective antigen delivery systems. Chitosan has been reported to act as both an adjuvant as well as a matrix for delivery systems Chitosan is a natural product produced predominantly from crab shell and commercially available preparations vary in molecular weight, degree of deacetylation and purity [9-11].

The membrane of bacteria having a lot of proteins showed good vaccine potential but problem was same as with the inactivated whole cell vaccine, therefore serious attempts for vaccine development to cope with this problem are required, Outermembrane proteins (OMPs) such as outermembrane vesicles (OMVs) was reported to have the ability to elicit a T-cell response in mice [12].

While OMPs of *Acinetobacter baumannii* was identified as a promising candidate for active and passive immunization based on humoral immunodominance during infection in mice [13, 14] showed that OMPs of *Acinetobacter baumannii* can stimulate an immune response by activate both humoral immunity (B-cell) and cellular immunity (T-cll) to produce antibodies and cytokines to cope with the pathogens known as multidrug resistant [15] showed that OMPs of *Acinetobacter baumannii* can stimulate specific immune response more than lipopolysaccharide (LPS) of sme bacteria because the proteins can bustle both humoral and cellular immune response. The aim of this study was to prepare an experimental nano vaccine of outer membrane protein for *Acinetobacter baumannii*.

Method

Collection of samples

One hundred twenty five (urine, wounds, burns) samples were collected from patients who attended to Maternity and Pediatrics Teaching Hospital, AL-Diwaniya Teaching Hospital, Central Health Laboratory, during the period from October 2013 to the January 2014.

Laboratory animals

Fifty Albino male rats (Rattus norvegicus) were supplied by the College of Veterinary Medicine in AL- Qadisiyah University. Their ages at the start of the experiments were (6-8) weeks. They were divided into groups control group (10 rats), OMP group (10 rats), OMP+Chitosan group (10 rats); was kept in a separate plastic cage.

Isolation and Identification of bacteria

This bacteria was isolated and identified according to [16] used Chromagar™ *Acinetobacter* according to company guidelines (CHROMagar France).

Biochemical Tests

The VITEK2 and API 20 kit used for biochemical tests according to the company guidelines (Biomerieux – France).

Outer Membrane Protein extraction

Extraction of bacterial outer Membrane Protein was done according to [17], then the Biuret test was carried out to evaluate the presence and quantity of the protein [18].

Preparation of Chitosan Nanoparticle

Chitosan was prepared according to [19].

Detection the optimum concentration of chitosan nanoparticles

Detection the optimum concentration of chitosan done according to [20].

Antigen loading on Chitosan Nanoparticles

Antigen was loaded on Chitosan nanoparticles according to [21].

Median lethal dose (LD50) of OMP

To determine lethal dose (LD50), various doses of OMP were prepared (100, 150, 200, 250, 300 µg/ml), 1ml from each concentration was injected (5 rats in each group) by intraperitonieum (i.p.) and the 50% lethal dose LD50 was determined by counting deaths during 5 days. Control group was injected by 1ml from phosphate buffer (PBS). A count of live and dead rats were used for the determination of median lethal dose [22, 2]. While, safety test was done according to [23, 12]. Further; sterility test was done according to [24, 12], and challenge test was done as illustrated in tables-1.

Blood samples collection

After 7 days from the last dose of treatment, 3ml of blood samples were collected from rats via intracardiac puncture under general anesthesia by using diethylether the collected blood samples were divided into two portions; first was treated with anticoagulant was used for the estimation the total and differential WBC count. The second portion left until clotting then centrifuge of supernatant to separate the serum which transferred to suitable plane tube for serological tests [24].

Total leukocytes count and Differential count of leukocytes (DCL)

These tests were done according to [13] to monitor blood WBCs of treated groups.

Passive haemagglutination test

This test was done according to [6] in attempt to quantitation of specific antibodies in treated groups.

Determination of Cytokines

Determination of (IL-2, IL-6, IFN-γ) according to the manufacture (KOMA biotech/ Korea) guidelines.

Statistical Analysis

The data were statistically analyzed using the statistical package SPSS version 10.0 for windows. The investigated parameters were presented in as mean ± standard error (S.E.), and differences between means were assessed by ANOVA, followed by LSD or Duncan test. The difference was considered significant when the probability (P) value was ≤ 0.05 [25].

Results

Survey of Acinetobacter baumannii

The positive bacterial cultures were 18.1% *Acinetobacter baumannii* and 81.9% non *Acinetobacter* spp. (Fig.1).

Determination the lethal dose (LD50%) of OMP for Acinetobacter baumannii When injected in five serial concentrations into the peritoneum of rats, it was found that the lethal dose of rats are about (266.5) µg /ml, as indicated in the (Table 2).

$$\text{Proportional distance} = \frac{50\% - \text{the percentage of dead rats at concentrations lower than } 50\%}{\text{The percentage of dead rats in the highest concentrations of } 50\% - \text{The percentage of dead rats at concentrations lower than } 50\%}$$

$$= \frac{50-37.5}{75-37.5}$$

$$= 0.33$$

$$\text{Proportional distance for } 50\% = \text{Proportional distance} \times (\text{The highest concentration of } 50\% - \text{The lower concentration of } 50\%)$$

$$= 0.33 \times (300 - 250)$$

$$= 16.5$$

$$\text{LD50\%} = \text{Proportional distance for } 50\% + \text{The lower concentration of } 50\%$$

$$= 16.5 + 250$$

$$= 266.5 \text{ µg / 1ml}$$

$$\text{Proportional distance} = \frac{50\% - \text{the percentage of dead rats at concentrations lower than } 50\%}{\text{the percentage of dead rats in the highest concentrations of } 50\% - \text{the percentage of dead rats at concentrations lower than } 50\%}$$

$$\frac{50 - 0}{66.66 - 5} = 0.75$$

$$\text{Proportional distance for } 50\% = \text{Proportional distance} \times (\text{The highest concentration of } 50\% - \text{The lower concentration of } 50\%)$$

$$= 0.75 \times (100 - 50)$$

$$= 37.5$$

$$\text{LD50\%} = \text{Proportional distance for } 50\% + \text{the lower concentration of } 50\%$$

$$= 37.5 + 50$$

LD50% of the Chitosan nanoparticles

When injected three serial concentrations of chitosan nanoparticle into the intraperitonieum of rats, it was found that the lethal dose of rats are about (87.5) µg / ml, which is between the concentrations 50 and 100 µg / ml after 5 days as shown in the (Table-3).

Antibody titer:

The obtained results showed that means of antibodies titers were increased significantly ($P \leq 0.05$) in the two immunized groups OMP + Chitosan (860±12.5) and OMP group (508±4.4) when compared with control group (Fig. 2).

Serum profile of cytokine:

Serum profile of some cytokines (IL-2 and IL-6 and IFN-γ) in sera of immunized rats with antigens and serum of control group by using the enzyme-linked immunosorbent assay (ELISA), showed remarkable changes.

Interleukin-2 (IL-2)

The Obtained results showed that there were a highly significant increment ($P \leq 0.05$) in concentrations of IL-2 of the group treated with OMP+Chito (830±2.9 pg/ml) in sera of treated rats , compared with the group treated with OMP (818±3.8 pg/ml) and control group (812±2.4 pg/ml) . Whereas, no significant differences in IL-2 concentrations of

OMP group (818±3.8 pg/ml) compared with control group (812±2.4pg/ml). As shown in (Fig. 3)

Interleukin6 (IL-6)

The Obtained results documented a highly significant increment (P≤ 0.05) in concentrations of IL-6 of the group treated with OMP+Chito (37±6.5 pg/ml) in sera of treated rats, compared with the group treated with OMP (31±5.1 pg/ml) and control group (22±3.2 pg/ml) .also significant differences in IL-6 concentrations of OMP group (31±5.1 pg/ml) compared with control group (22±3.2pg/ml). As shown in (Fig. 4).

Interferon Gamma (IFN-γ)

The Obtained results showed that there were no significant changes (P≤ 0.05) in concentrations of IFN-γ of the group treated with OMP+Chito (1.5±1 pg/ml) in sera of treated rats , compared with the group treated with OMP (1.3±0.8 pg/ml) and significant increment compared with the control group (1.15±0.3 pg/ml) . Whereas, no significant differences in IFN-γ concentrations of OMP group (1.3±0.8 pg/ml) compared with control group (1.15±0.3pg/ml) Fig. 5.

Total leukocytes count (TLC)

Documented data of the current study showed that rats treated with OMP group (11.200±300 cells/ mm3. Blood) which was non-significant as compared with the control group (9.500±200 cells/ mm3. blood).But rats that treated with OMP+Chito (18.200±500cells/ mm3. blood) showed a significant increase in total leukocytes counts as compared with the OMP group (11.200±300 cells/ mm3. Blood) and control group (9.500±200 cells/ mm3. blood) Fig. 6.

Differential count of leukocytes (DCL)

The obtained results showed that rat's which treated with OMP and OMP + Chitosan showed no significant increase in percentage of neutrophils in compared to the control group. rats that treated with OMP + Chitosan group showed a significant increase in the percentage of lymphocytes as compared to the control group and with OMP showed significant changes in the percentage of lymphocytes as compared to the control group. And no significant increase in the percentage of eosinophils, monocytes and basophiles between all groups (Table 4).

Group	No. of animals	Treatment		
		First dose	Boosting dose	Challenge dose
		First week	Third week	Fifth week
OMP	5	266.5µg+0. 5ml	266.5µg+0. 5ml	533µg+1ml
OMP+Chitosan	5	266.5+85µg/0. 5ml	266.5+85µg/0. 5m	533+170µg/1ml
Control	5	0. 5ml (PBS)	0. 5ml (PBS)	0. 5ml (PBS)

Table -1

The design of the challenge test.

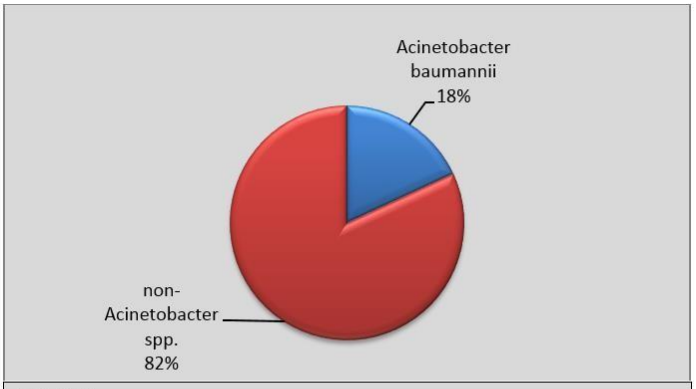


Figure 1.

Percentage of *Acinetobacter baumannii* and non *Acinetobacter* spp.

Concentration Of OMP µg / 1ml	No. of Rats			The cumulative number of rats		Total cumulative numbers	Percentage of dead rats
	Treated	Dead	Live	Dead	Live		
300	5	3	2	6	2	8	75%
250	5	2	3	3	5	8	37.5%
200	5	1	4	1	9	10	10%
150	5	0	5	0	14	14	0%
100	5	0	5	0	19	19	0%

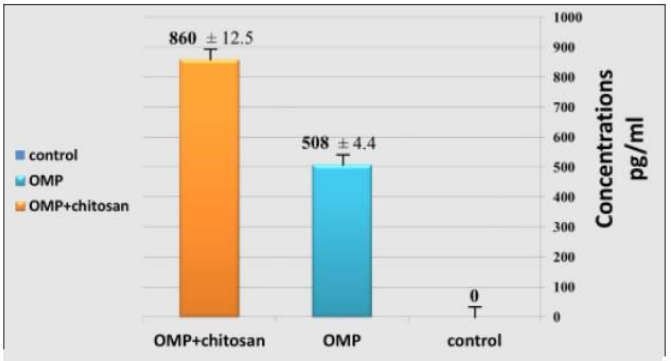
Table-2

Lethal dose (LD50%) of the OMP for *Acinetobacter sp*

Concentration Of chitosan nanoparticle µg / 1ml	No. of Rats			The cumulative number of rats		Total cumulative numbers	Percentage of dead rats
	Treated	Dead	Live	Dead	Live		
150	3	3	0	5	0	5	100%
100	3	2	1	2	1	3	66.66%
50	3	0	3	0	4	4	0%

Table-3

Lethal dose (LD50%) of chitosan nanoparticle



Figer (2) means of antibodies titers

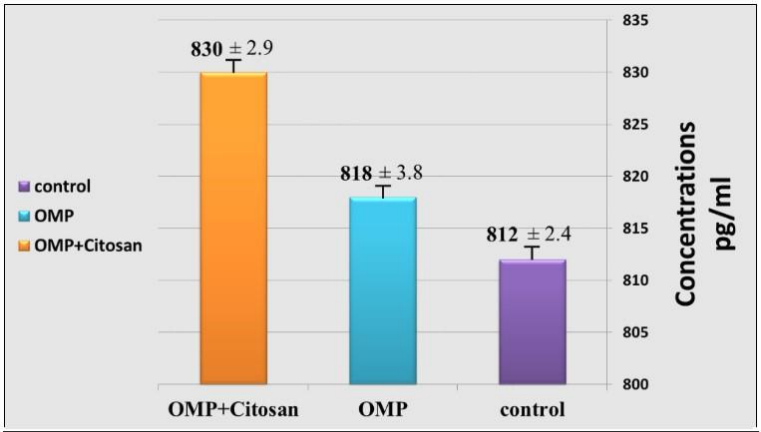


Figure 3.

Concentrations of IL-2 in rats treated with different antigens.

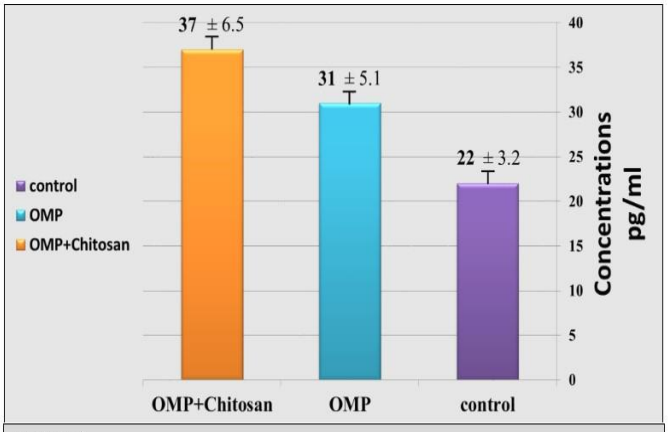


Figure 4.

Concentrations of IL-6 in rats treated with different antigens.

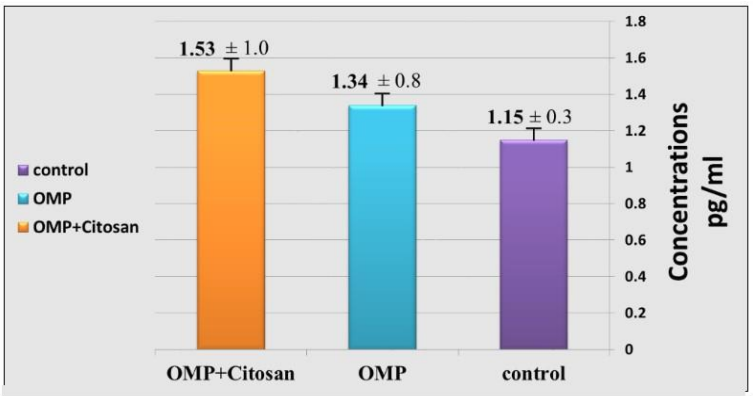


Figure 5.

Concentrations of IFN-γ in rats treated with different antigens.

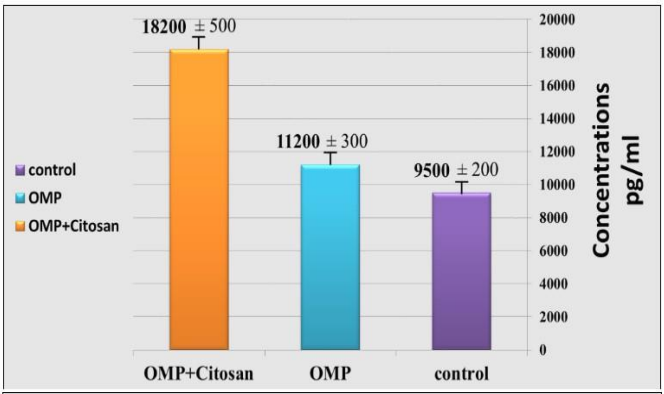


Figure 6.

Total WBCs counts in rats treated with different antigens

Groups WBCs	Percentage of Differential WBC (%) Mean ±SD				
	Neutrophils	Lymphocytes	Eosinophils	Monocytes	Basophiles
Control	51.9±3.1	41.6±2.8	2.1±0.6	3.6±1	0.8±0.2
OMP	52±2	43.4±2.2	1.1±0.2	3.3±1.3	0.2±0.07
OMP+Chitosan	49.2±2.6	46±2.4	1.3±0.3	3.1±1.3	0.4±0.1

Table-4

Differential count of leukocytes in rats treated with different antigens.

Discussion

The incidence of *Acinetobacter* infections has risen significantly and continuously worldwide. This bacterium became important nosocomial pathogens. MDR strains are difficult to treat and associated with significant morbidity and mortality [7]. Although this bacterium is an organism of low virulence, it is able to cause serious infections [26].

Hospital environment contamination with *Acinetobacter* species apparently occurs quite frequently because the organism can survive in dry conditions for a prolonged period [27]. They are able to survive on dry particles and dust up to ten days and more than four months on both moist and dry surfaces such as rubbers, ceramics and various types of medical equipment Furthermore, *Acinetobacter* able to form biofilm that it from environmental conditions [28], that may explain its ability to survive in nosocomial environments and to cause device-related infections in compromised patients [29].

The conditions for formation of high yield nanoparticles of a particular nanometric size may vary significantly depending on the purity, acid salt, and molecular weight of the Chitosan used. These features include ease of formation, a very homogeneous, a positive surface charge that can be conveniently modulated, a good capacity for association with peptides, proteins, vaccines, oligonucleotides, and plasmids, and, finally, release of the associated molecules at different rates depending on the composition of the particles [30]. The increasing of absorbance that indicates the loading of antigen on nanoparticles, in triple OMP antigen with chitosan the absorbance was increased, this indicates the ability of chitosan nanoparticle to carry more than one of antigen.

Antigen is loaded with the nanoparticles when dissolved in alkaline sodium tripolyphosphate solution. This is attributed to the ionic interaction between the sodium tripolyphosphate solution and chitosan upon mixing. Other forces, including hydrogen bonding and hydrophobic forces, could be involved in the association process [31]. Antigen loaded nanoparticles showed a positive charge. This positive charge is in favor of the vaccine formulation, and the chances of positively charged nanoparticles are higher than negative charge or neutral for phagocytosis [32].

The amine groups of chitosan can be changed under weak acid condition into the – NH3 + anion, which may interact with the cell wall of bacteria and prevent the growth of the microorganism [33, 34], found possibility of loading recombinant Leishmania superoxide dismutase B1 (SODB1) on chitosan nanoparticles in BALB/c mice, SODB1-loaded nanoparticles showed a positive charge. The enhancement of cellular immune response after OMPs immunization, can be explained by the fact that OMPs are biological active molecules that activate immune cell via toll – like receptors, Cytokines such as IL-2 are known to mediate the inflammatory response. They act through complex mechanisms [35].

IL-2 is a key cytokine with broad-spectrum and crucial immunomodulatory activities. One such activity is to enhance the function of T cells, macrophages and natural killer cells, thereby inducing T cells to generate interferons and activating Th cells [36]. IL6 are known to mediate the inflammatory response. They act through complex mechanisms. IL-6 is produced by T cell and many other cell types and induces acutephase protein synthesis , T cell activation , and IL-6 production and stimulates B

cell Ig production and hematopoietic progenitor cell growth , the result showed that IL-6 that promotes the B-cell to differentiation and produce a high number of immunoglobulin , the advances studies that provide IL-6 is a growth factor for EBVimmortalized B-cell and extends these results, demonstrating that IL-6 transcriptionally activities a number of growth –responsive genes [37]

IFN-γ is an important immune modulator secreted from activated T lymphocytes and natural killer (NK) cells following viral or parasitic infections. Leukocytes are considered as the active cells in starting out the functions of the immune system, both non-specifically and specifically, and their count may give a general picture about the function of the immune system (Lydyard & Grossi, 1998). Due to these diverse immunological functions, the normal counts of leukocytes (total and differential) can be deviated by infections [38].

In agreement with such theme, both counts (total, differential) of leukocytes were deviated in the present study, but the deviation was subjected to the group investigated and whether control or post-challenged. In a more recent estimation, the range was 5000-12000 cells/ mm3.blood [39]. An observation that may suggest that LPS may able to enhance the adaptive immunity. Such suggestion has some support from a study carried out by, While in rats immunized with OMP + Chitosan, total WBCs counts were recorded highly significant increase in post-challenged animals as compared with control rats, because the Chitosan can be activate of leukocytes, this agreement with previous studies like [40]. The differential count of WBCs was significantly increased especially the percentages of neutrophils in immunized animals with OMP as compared with control rats.

This increment related to the function of neutrophils that is carrying out phagocytosis in the innate immune system [41]. But, when used chitosan with OMP we noticed elevation the percentage of neutrophils in primed animals more than in the case using OMP alone as compared with control rats because the ability of chitosan nanoparticles to play an active role in recruiting a variety of immune cells (monocytes, macrophages, neutrophils, natural killer cells, dendritic cells, and memory T cells) to the site of infection or inflammation [42-45], demonstrated that the wild derived inbred mouse strain SPRET/Ei is resistant to OMP, although an enhanced immunity was observed. In addition, it has been demonstrated that such resistance in SPRET/Ei mice

to S. typhimurium infection is associated with increased leukocyte counts reaching the upper limit of the range in the circulation and enhanced neutrophils influx into the peritoneum during the course of infection [46].

On the other hand, the immunized rats with OMP + Chitosan, lymphocyte percentages was highly significant increase in post-challenged animals as compared with control rats. This results were due to the ability of chitosan to increase both cellular and humoral immune responses and elicited a balanced Th1/Th2 response because lymphocyte represent the humoral and cellular arms of specific immunity [22]. This is an agreement with report by author [47]. Monocytes percentages was highly significant increase in post-challenged animals as compared with control rats, such as in vaccinated rats with OMP, also elevation percentage of Monocytes in vaccinated rats with OMP + Chitosan, as compared with control rats. These results were due to the ability of Monocytes to increase when lymphocyte increment [48].

Competing interests

The authors declare that there is no conflict of interest.

Authors Contributions

All authors wrote, read and approved the final manuscript.

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