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Designing an enzyme-linked immunosorbent assay for detection of anti-penicillin antibodies levels in various species of animals in Khuzestan province, Iran

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Abstract

The penicillin allergy is being increasingly recognized as a significant public health problem. Immunological responses to penicillin and other beta-lactam antibiotics are classified as immediate and non-immediate responses. This research aimed to develop an enzyme-linked immunosorbent assay (ELISA) for the detection of the reactive antibody value against penicillin in various species of animals. The serum samples were collected from nine species (forty mature animals in each species) including horse, dog, goat, sheep, buffalo, cattle, donkey, chicken, and fish. The concentrations of total antibody and immunoglobulin M (IgM) against penicillin were detected using an in-house ELISA test. The total anti-penicillin antibodies concentration from high to low in animals was as chicken, horse, fish, donkey, dog, goat, sheep, buffalo, and cattle, respectively. In cattle and sheep, the level of anti-penicillin IgM (APM) was significantly higher than non-IgM antibodies (APNM). Moreover, levels of APNM were very low in chicken and fish serums; no difference was seen regarding these values in buffalo and goat. The other species had significantly lower APM than the APNM. The ani-penicillin antibody levels in the noted animals were successfully detected using the developed ELISA. Most of the species have anti-penicillin antibodies; however, they have reactive antibodies with differences in levels and isotypes.

Introduction

The administration of drugs may induce undesirable immune responses; according to the type and severity of the immune responses and have several effects including asymptomatic and adverse clinical signs. The creation of anti-drug antibodies (ADAs) may reduce the efficacy and safety of drugs. The ADAs can also impact on bioreactivity and alter the pharmacokinetics and pharmacodynamics of drugs.[1] The correlation between dose and drug concentration in serum or tissue is another factor that could be affected in this situation.[2] The anti-drug antibodies affecting on drug clearance rate are classified as clearing antibodies, sustaining antibodies or neutralizing antibodies. It's noticed that the cross-reaction of ADAs with host proteins or their ability in the onset of anaphylactic reactions may cause damage to the host organs.[1] Despite the protective role of immunoglobulin G (IgG) in allergic diseases, after high dose administration of drugs, the circulatory IgG and IgM may develop an immune complex and cause the third class of hypersensitivity reactions. Also, it may cause cell cytotoxicity reaction induction,[3] when the antigen is attached to the cell membrane surface.

Detection of the immunogenicity and induction of neutralizing and non-neutralizing ADAs production are critical factors in the safety of the drug assessment.[4]

Drugs usually do not induce immune responses because of their small molecular size. Beta-lactams antibiotics are the common causes of immediate and late hypersensitivity reactions to drugs.[5] The β-lactam antibiotics can bind to the host protein and act as a hapten-carrier complex. They may undertake isomerization to penicillanic acid and may bind to other molecules

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stimulating the immune system.[6] The immediate symptoms of penicillin allergy are mediated by IgE; while non-immediate allergies are provoked by the other antibody isotypes or T lymphocytes. Prediction of these allergic reactions is usually difficult; especially if the common allergy tests become negative or in a situation of normal clinical history.[7] The presence of IgG and IgM antibodies against penicillin may cause hypersensitivity reactions. The outcome of penicillin injection could include any types of hypersensitivity reactions. This study was aimed to design an enzyme-linked immunosorbent assay (ELISA) for measuring the levels of total antibody and IgM against penicillin in various species of animals.

Materials and Methods

The utilized Tween-20, beta-2mercaptoethanol, sulphuric acid, blood agar base were provided from Merck (Kenilworth, USA). The purchased material from Sigma (Taufkirchen, Germany) are included bovine serum albumin (BSA), glutaraldehyde (GA), penicillin G potassium, diethylaminoethyl cellulose (DEAE-C), sepharose 4B and ethylenediaminetetraacetic acid (EDTA). The horseradish peroxidase-conjugated goat anti-rabbit IgG (HRP Affi Pure Goat anti-Rabbit IgG Fc) provided from ImmunoChemistry Technologies (Bloomington, USA). Other salts and chemical materials which used for the preparation of the buffers were purchased from Merck, excluding Tannic acid (Fluka, Gillingham, UK) and Tetramethylbenzidine (TMB; Raha Zist Padtan, Ahvaz, Iran).

Sample collection. The serum samples were collected from nine species (forty mature animals in each species) including horse, dog, goat, sheep, buffalo, cattle, donkey, chicken, and fish. Samples were randomly selected from different regions of Khuzestan province, Iran. All experimental protocols were approved by the Research Program Animal Care and Use Committee of the Shahid Chamran University of Ahvaz, Ahvaz, Iran (Act No. 960121).

Penicillin conjugation. Molecules of penicillin and BSA were cross-linked together using GA.[8] One mL penicillin G potassium (10.00 mg mL^{-1}) in a solution containing 0.50 M ethylene EDTA was incubated with 1.00 mL of 5.00 mM GA for 3 hr with stirring. One hundred µL of BSA (5.00 mg mL^{-1}) in phosphate-buffered saline (PBS) was added to this mixture and the whole mixture was incubated for 24 hr with slow stirring at room temperature. The coupling mixture was dialyzed for 72 hr against several changes of PBS at 4.00 ˚C.

Conjugation analysis. The molecular weight of the conjugates was defined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The presence of penicillin in the produced conjugate was tested using antibiogram method. The *Bacillus anthracis* was cultured in blood agar plate media. The penicillin G and PBS were loaded to the other plates as positive and negative controls, respectively. The presence of penicillin in produced conjugate was interoperated with an inhibitory zone formation.

Immunization. The produced penicillin-BSA conjugate was used to immunize two rabbits. The immunization methods were the same as those described previously. The immunized blood was directly collected and allowed to clot; serum was pipetted out and stored at 4.00 ˚C. After the confirmation of good titers checked by passive hemagglutination. Briefly, sheep red blood cells (SRBCs) were washed three times with phosphate-buffered saline (PBS), adjusted to a concentration of 2.50% in PBS and reacted with the same volume of 0.001% tannic acid in PBS. The mixture was incubated for 30 min at room temperature. Then, the treated SRBCs have washed again and re-suspended in PBS at a concentration of 2.50%. The sensitized SRBCs reacted with the same volume of penicillin G in the concentration of 10.00 $mgmL^{-1}$ and incubated for 4 hr at 37.00 ˚C. After incubation, the cells were washed three times with PBS and re-suspended in PBS.[9] The penicillin-coated SRBCs 1.00% was added to microtiter plate wells containing serially diluted serum of the immunized rabbits.

Purification of specific antibodies against penicillin. The IgG fraction was extracted by performing ion-exchange chromatography on the DEAE-C column according to Hay and Westwood.[9] The specific IgG was purified by affinity chromatography according to Khoobdel *et al*. in which the column was prepared by conjugation of 20.00 mg penicillin to 5.00 mL of activated CH Sepharose 4B.[10] The 5.00 mL of CH Sepharose 4B was activated using cyanogen bromide according to the method of Nishikawa and Bailon.[11] After three sets of washing, the penicillin G 20.00 mg was added to the mixture and rotated for 4 hr at room temperature. The total IgG was added to the washed Sepharose 4B and rotated again for 60 min. The prepared mixture (sepharose 4B- penicillin G- antibodies) was placed on a 5 mL column. After washing the column with PBS, the specific antibody was eluted from the column with 0.10 M glycine (pH 2.50) and neutralized immediately by adding an appropriate amount of 1.00 M Tris-pH 9 to each fraction.

ELISA for antibody titer against penicillin. Flat-bottomed ELISA plates were coated overnight with conjugated penicillin (1.00 µg per well) in 1.00 M sodium bicarbonate with pH of 9.60. The plates were then washed three times with PBS /0.05% Tween-20 (PBST) and blocked for 2 hr at 37.00 ˚C with 2.00% Tween-20. A part of samples was treated with 0.10 M beta-2mercapto-ethanol and the samples were dispensed in duplicate wells. The plate was incubated for 1 hr at room temperature. After washing three times with PBST, the purified specific rabbit's IgG against penicillin (2.00 µg per well) was added to each well. After incubation and

washing as before, the horseradish peroxidase-conjugated goat anti-rabbit IgG (HRP Affi Pure Goat anti-Rabbit IgG Fc) was diluted as 1/10000 and added to each well. The plate was incubated for the next 1 hr at room temperature. After washing, the substrate solution of TMB was added to the wells. The reaction was stopped with 50.00 µL of 2.00 M sulfuric acid before reading the absorbance at 450 nm. The standard curve was created using a serial dilution of specific IgG instead of the tested samples. The individual-specific antibody against penicillin (A; µg mL^{-1}) was calculated as below:

A = 2 – Value of specific IgG binding to penicillin in each well

Statistical analysis. In this study, the differences between species or the target isotypes levels were assessed using repeated measure ANOVA. In all statistical analyses, the *p*-value of less than 0.05 was considered significant.

Results

The penicillin was conjugated to BSA by a short cross-linker reagent (GA). The coupling efficiency of conjugation was confirmed using SDS-PAGE, antibiogram, and passive hemagglutination test. The presence of the anti-penicillin antibody was detected by ELISA. The results (Table 1) showed that chicken, horse, fish, and donkey species respectively had higher levels of total anti-penicillin antibody than the other species. A significant difference, regarding the non-IgM level, was observed between horse, donkey and dog, and other species. The highest IgM anti-penicillin level was detected in chicken and fish samples.

Table 1. The concentration (µg mL^{-1}) of the total antibody, non-IgM, and IgM anti-penicillin in various species. The antibody levels were detected using an in-house ELISA. The animals were sorted in rows according to the highest level of the total antibody against penicillin. Data are presented as mean ± SD.

Species	Total antibody	Main non-IgM	Main IgM
Chicken	1.85 ± 0.13^{a}	0.16 ± 0.09^{c}	1.79 ± 0.19^{a}
Horse	1.70 ± 0.15^{a}	1.40 ± 0.65^{a}	0.35 ± 0.15^{c}
Fish	1.65 ± 0.19^{a}	0.00 ± 0.00^{d}	1.65 ± 0.19^{a}
Donkey	1.64 ± 0.27^{ae}	1.02 ± 0.38^{ae}	0.62 ± 0.14^{b}
Dog	1.30 ± 0.66^{a}	1.34 ± 0.65^{a}	0.05 ± 0.01^{d}
Goat	0.70 ± 0.48^{b}	0.34 ± 0.12^{cf}	0.36 ± 0.12^{c}
Sheep	0.65 ± 0.26^{b}	0.12 ± 0.08^{c}	0.53 ± 0.10^{b}
Buffalo	0.49 ± 0.33^{b}	0.28 ± 0.11^{c}	0.21 ± 0.11^{c}
Cattle	0.42 ± 0.34^{b}	0.12 ± 0.18^{cf}	0.30 ± 0.15^{cf}

Different superscript letters show significant difference ($p \leq 0.05$) on the level of anti-penicillin antibody between different species.

Discussion

The anti-drug antibodies are measured by enzymatic immunoassay or bridging assays. The most recent research reported the ELISA assay as a suitable approach for this purpose.[12] Previously, the US Food and Drug Administration (FDA) has suggested that the ADA screening assay sensitivity is around 250-500 ng mL^{-1} to be able to pick up clinically relevant immunogenicity. Recently, FDA guidance recommended the assay sensitivity level of 100 ng mL^{-1} as they have observed clinically relevant responses at this level.[13] The developed ELISA in this study at least has sensitivity equal to 50.00 ng.

According to Thway *et al*. classification,[1] based on non-IgM titer against penicillin, the species can be classified as high titer included horse, dog, and donkey; medium titer comprising none of the animal species; low titer including goat, buffalo, chicken, cattle and sheep and negative titer including fish. The humoral immune responses to penicillin were investigated by Lee *et al*.[14] and they have reported a positive correlation between the total dose of injection and the level of IgG titer. The complement activation occurred by IgG attachment to allergen-IgE complexes may affecting processing and presentation of the allergen; thereby IgG level can control allergic reactions.[15] The higher IgG levels specific to various allergen components were found in sensitive subjects[16] or patients with negative skin tests but typical symptoms;[17] the people with an adverse reaction to penicillin have higher levels of the IgM and IgG anti-penicillin. The current experiment suggested that chicken, horse, fish, and donkey are on a higher risk to develop a non-immediate hypersensitivity reaction following penicillin injection.

The anti-penicillin antibody titers were detected in all analyzed species. In addition to drug consumption, most of the food and drink products are also polluted with penicillin. The isotype of the produced antibodies is dependent on the dose and number of penicillin injections. A single injection or first administration would produce IgM with low affinity; however, several injections induce high-affinity IgGs. The repeated injection may cause autoimmune diseases by breaking the immune tolerance to self-proteins.[18] The immune responses were stimulated in most experimental animals undergoing a long-term treatment during chronic therapeutic studies; also, production of the ADAs and inhibition of the pharmacologic effects have occurred in this situation.[19]

The clearance of the immune complex affected by anti-drug IgG and IgM, whereas IgG antibody may interfere with drug activity and IgM with low affinity being separated from the drug and has no considerable effects.[20] The decrease of the drug -ADAs complex clearance from circulation often occurring during long-term and multiple-dose treatments can lead to embellished pharmaco-dynamics effects.[20]

This experiment detected different titers against penicillin in animal species; the immunogenic reaction to drugs is affected by several factors including genotype, age, drugs component, uptake by immune cells and modification in formulation or manufacturing process, in addition to the other factors such as accompanied

treatment, route of administration, formulation, dose, and frequency of dosing.[1]

The current study classified various species of animals according to the total and IgM anti-penicillin antibody levels. The higher level of non-IgM antibody predicts the higher risk for immune complex or cell cytotoxicity hypersensitivity reactions. Also, the existence of the significant anti-penicillin antibodies may cause pharmacokinetics and pharmacodynamics effects.

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Conflict of interest

Authors declare that have no conflict of interest.

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