

# Immunoglobulin G responses in 21 dogs with skin diseases to antigens from different isolates of *Staphylococcus intermedius*

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**The aim of this study was to characterise the immunoglobulin G (IgG) response in 21 dogs with or without pyoderma to antigens from six isolates of *Staphylococcus intermedius*. The staphylococcal proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis, transferred electrophoretically on to a membrane and subjected to immunoblotting with the dogs' serum. Gels containing separated proteins from the six isolates revealed 29 to 33 distinct bands with molecular weights ranging from 20 to 230 kDa. All the dogs' sera contained IgG that recognised 12 to 24 bands (mean 17), regardless of whether the dogs had pyoderma. The recognised proteins had molecular weights ranging from 20 to 198 kDa but the majority had molecular weights below 75 kDa. The most intense band in all six isolates had a molecular weight of 28 to 29 kDa. The antibody responses to the six isolates were essentially similar except that there were significantly more bands in the response to isolate 2 than to isolate 6, and occasional differences in the intensity of individual bands. All 21 dogs mounted an IgG response to multiple antigens in *S. intermedius*, which differed only marginally between the six isolates. This lack of variation provides evidence that the host's response to different isolates of *S. intermedius* is not a major factor in canine pyoderma.**

*Staphylococcus intermedius* is the organism most frequently isolated from dogs with pyoderma (Cox and others 1984) and is variably regarded as a transient or resident cutaneous microorganism (Allaker and others 1992). It can frequently be cultured from the nares, oropharynx and anal ring of healthy dogs and is therefore thought to be part of the resident flora in these regions (Devriese and De Pelsmaecker 1987, Allaker and others 1992). The pathogenesis of staphylococcal pyoderma is unclear but it is generally accepted that primary pyoderma is very uncommon (Ihrke 1987, Allaker and others 1992) and the infection is thought to become established by the opportunistic growth of this nomadic organism on skin affected by an underlying disease process, for example, hypersensitivity disorders, endocrinopathies, ectoparasite infestations or primary cornification defects. Once pyoderma has developed, it rarely resolves spontaneously, and it commonly recurs, suggesting that dogs may not mount a protective immune response against the organism.

The interaction between *S. intermedius* and the immune system of dogs has not been extensively investigated. It is known that dogs mount an immunoglobulin G (IgG) response to *S. intermedius* (Shearer and Day 1997; R. E. W. Halliwell, personal communication), but the specific antigens that are recognised have not been well characterised. It is not known whether IgG responses differ between isolates of the organism. Previous studies have shown that there is no significant strain variation in the expression of virulence factors by *S. intermedius* but it is not known whether this is also the case for antigens. The aim of this study was to determine the patterns of antigen recognition by IgG in dogs with or without pyoderma to six different isolates of *S. intermedius*. It was hypothesised that, as with the expression of virulence factors, the IgG responses would not differ markedly between the isolates because such differences might be expected to result in variable pathogenicity of the organism.

## MATERIALS AND METHODS

### Serum samples

Serum samples were collected from 21 dogs with various skin diseases and stored at  $-70^{\circ}\text{C}$  until used. The dogs were diagnosed with atopic dermatitis, *Cheyletiella* species infestation, sarcoptic mange, *Malassezia* dermatitis, hypothyroidism, hyperadrenocorticism, mast cell tumour, pemphigus

foliaceus, pemphigus vulgaris, seasonal flank alopecia and zinc-responsive dermatosis. Nine of the dogs had evidence of active staphylococcal pyoderma as indicated by suggestive lesions, for example, papules, pustules or epidermal collarettes, and the presence of inflammatory cells and cocci on cytological specimens. Stored samples of serum were also available from eight dogs that had no history or physical signs of skin disease; they had been obtained from rescue dogs that were euthanased for behavioural reasons.

### *S. intermedius* isolates

Six different isolates of *S. intermedius* obtained from dogs with either pyoderma or otitis were chosen empirically and used for these studies. *S. intermedius* was identified by using the coagulase test, the DNase test, hyaluronidase production and haemolysin production on sheep blood agar. The isolates had been stored on plastic beads (Mast Group) at a temperature of  $-70^{\circ}\text{C}$  for up to three years.

### Preparation of extracts

Individual beads were selected from the frozen samples and rolled out on horse-blood agar plates which were cultured at  $37^{\circ}\text{C}$  overnight. In order to acquire sufficient numbers of organisms for the preparation of extracts, each isolate was subcultured on to 30 horse-blood agar plates and incubated overnight at  $37^{\circ}\text{C}$  until the colonies were confluent. The bacteria were then harvested and suspended in phosphate-buffered saline (PBS), pH 7.4 in which they were washed three times by centrifugation at 500 g for 15 minutes at  $4^{\circ}\text{C}$ , removal of the supernatant and resuspension in PBS. After the last washing cycle, the bacteria were resuspended in an extraction buffer containing 125 mM ammonium bicarbonate (Sigma) and the protease inhibitors 20 mM  $\epsilon$ -aminocaproic acid (Sigma), 5 mM ethylenediaminetetraacetic acid (Sigma) and 1 mM phenylmethylsulphonyl fluoride (BDH). The best extraction of proteins was obtained by disrupting the organisms mechanically by vortexing them with glass beads (0.4 mm, 40 mesh; BDH) for 10 minutes (data not shown). The cell suspensions and beads were then centrifuged at 6000 g for five minutes and the supernatant was collected. The supernatant was filtered through a 0.2  $\mu\text{m}$  syringe filter (Nalgene; Nalge Nunc International) to sterilise it. An aliquot of the extract from each isolate was incubated on blood agar for three days at  $37^{\circ}\text{C}$  to confirm the absence of bacterial growth. The protein content of each staphylococcal extract was measured by

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using a BCA protein concentration assay (Pierce Chemical Company) according to the manufacturer's instructions.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

This procedure was carried out as described by Laemmli (1970), using a 10 per cent separating and a 4 per cent stacking gel in a discontinuous buffer system containing 0.025M Tris (Sigma), 0.2M glycine (Fisher Scientific) and 0.1 per cent SDS (Fisher Scientific). For each gel, 15 µl each of the six *S. intermedius* extracts was diluted 1:1 with reducing sample buffer containing 5 per cent β-mercaptoethanol (Sigma), 1 per cent glycerol (BDH), 0.2 per cent SDS, 0.6 per cent Tris and bromophenol blue (Sigma) and heated for five minutes at 95°C together with a broad range molecular weight standard (Bio-Rad); 15 µl of each of the six extracts and 5 µl of the molecular weight standard were then added to separate wells in the gel which was electrophoresed at 200 V for 40 minutes.

Gels not used for immunoblotting were stained with 0.25 per cent Coomassie blue solution (Coomassie blue R; BDH) in 10 per cent acetic acid (Fisher Scientific) for five to 10 minutes and destained with PAGE destain (10 per cent acetic acid, 45 per cent methanol; Fisher Scientific) for two to three hours with several solution changes to obtain clean bands and a clear background.

Electrophoretic transfer

The separated proteins were transferred from the gel to a polyvinylidene difluoride microporous membrane (Immobilon-P Transfer Membrane; Millipore Corporation) in a Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) as described by Towbin and others (1979a) and modified by Burnette (1981). The gel was laid on a similar sized piece of membrane that had been immersed in 100 per cent methanol for three seconds and subsequently equilibrated in a transfer buffer containing 3.03 per cent w/v Tris, 14.2 per cent w/v glycine, and 20 per cent v/v methanol (Fisher Scientific). The gel and membrane were 'sandwiched' between four layers of chromatography paper (Whatman) pre-wetted in transfer buffer and the transfer was run for one hour at 80 mA. The quality of transfer was checked by staining gels and molecular weight standards blotted on to the membrane with Coomassie blue as described above.

Immunoblotting with anti-canine IgG

Membranes containing the transferred proteins of the six *S. intermedius* extracts were blocked with 5 per cent skimmed milk in Tris buffered saline (TBS) containing 0.02M Tris and 0.5M sodium chloride, probed with the serum of one dog per membrane and incubated with goat anti-canine IgG conjugated with horseradish peroxidase (Bethyl Laboratories). Preliminary experiments showed that serum dilutions of 1:100 and goat anti-canine IgG at a dilution of 1:5000 gave the best results (data not shown). The membranes were developed with a 3,3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories). Each blocking and incubation step lasted one hour except the development in DAB, which took one to two minutes. Between each incubation step the membranes were washed three times for five to 10 minutes in TBS with 0.05 per cent Tween 20 (Fisher Scientific).

To ensure the specificity of the goat anti-canine IgG antibody, purified canine IgG, purified canine IgE and dog serum were probed as described above. No cross-reactivity was observed with canine IgE and one distinct band was observed at the appropriate level with both the canine IgG and the dog serum. Specificity against IgM and IgA was provided by the manufacturer's data sheet. To prove that the anti-canine IgG did not bind non-specifically to sta-

TABLE 1: Numbers and molecular weights (kDa) of the protein bands from six isolates of *Staphylococcus intermedius* separated by SDS-PAGE, stained with Coomassie blue, and the data generated by digital image analysis

|                                     | Isolate 1 | Isolate 2 | Isolate 3 | Isolate 4 | Isolate 5 | Isolate 6 |
|-------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Number of bands                     | 33        | 32        | 33        | 33        | 30        | 29        |
| Molecular weight range (kDa)        | 20-152    | 20-150    | 20-146    | 20-144    | 23-234    | 23-230    |
| Most intense band (kDa)             | 56        | 50        | 49        | 25        | 57        | 26        |
| Band of 2nd highest intensity (kDa) | 50        | 56        | 55        | 54        | 25        | 41        |
| Band of 3rd highest intensity (kDa) | 43        | 58        | 42        | 49        | 55        | 55        |
| Band of 4th highest intensity (kDa) | 58        | 42        | 57        | 38        | 41        | 43        |

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

phylococcal proteins, control experiments were performed in the absence of canine serum. No bands were observed in these blots.

Digital image analysis

In order to analyse the blots, the lane containing the molecular weight standards was cut off the edge of the membrane, stained with Coomassie blue and realigned with the membrane next to the stained IgG bands. A digital image was acquired for each membrane under identical conditions and settings (Nikon Coolpix 995; Nikon). The images were analysed by using Digital Science 1D Analysis Software (Kodak) which detects bands on the blots, analyses their position relative to the molecular weight marker, and gives a semi-quantitative assessment of the intensity of the bands.

Statistical analysis

The data were analysed by using GraphPad Prism version 3.00 for Windows (GraphPad Software). The numbers of bands for each isolate were compared by using a one-way repeated measures analysis of variance with a Tukey's multiple comparison post-test. The intensity of the bands and the molecular weight range for the six different isolates were not distributed normally and were analysed by a Kruskal-Wallis analysis with a Dunn's multiple comparison post-test if significant differences were found. P<0.05 were considered to be significant.

RESULTS

*S. intermedius* proteins separated by SDS-PAGE gel

The staphylococcal proteins separated by SDS-PAGE from the six different isolates and stained with Coomassie Blue had a similar profile (Fig 1). The numbers of distinct bands, their molecular weight range and the highest intensity bands are shown in Table 1. The most intense bands appeared either in the 49 to 57 kDa range (isolates 1, 2, 3 and 5) or in the 25 to 26 kDa range (isolates 4 and 6).

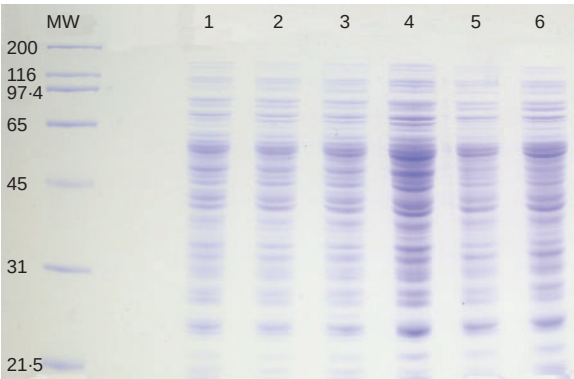
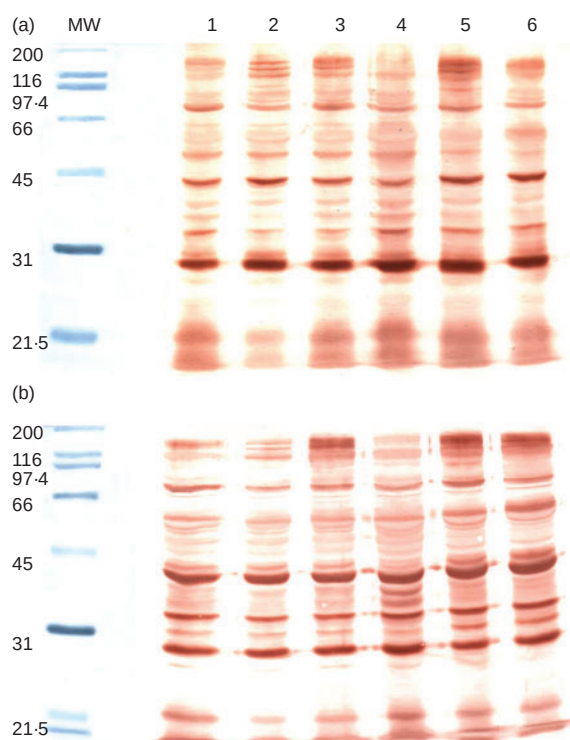


FIG 1: Coomassie blue-stained sodium dodecyl sulphate polyacrylamide gel electrophoresis of six isolates of *Staphylococcus intermedius*. MW Bio-Rad broad band protein molecular weight marker, Lanes 1 to 6 Isolates 1 to 6

**FIG 2:** Diaminobenzidine-developed immunoblots of separated staphylococcal antigens from the six (1 to 6) extracts of isolates of *Staphylococcus intermedius*. (a) Typical blot incubated with canine serum and probed with anti-canine immunoglobulin G from a dog with pyoderma, (b) a blot from a dog that did not have pyoderma. MW Bio-Rad broad band protein molecular weight marker



### IgG response to staphylococcal antigens

An analysis of the immunoblots showed that there was an IgG response to multiple proteins of different molecular weights with all six isolates of *S. intermedius*, and that an IgG response was present in the dogs whether or not they had pyoderma (Fig 2). To compare the isolates, all the data were therefore analysed as a single group.

**Numbers of bands recognised** The mean numbers of bands in isolates 1 to 6 were 17, 18, 17, 18, 18 and 16, respectively. The minimum number of bands recognised was 12 (isolates 1, 2, 3, 5 and 6) or 13 (isolates 4), and the maximum number of bands recognised was 23 (isolates 1, 2 and 3) or 24 (isolates 4, 5 and 6). There were significant differences among the six isolates ( $P < 0.05$ ) which were attributable to a significantly larger number of bands being recognised in isolate 2 than in isolate 6 ( $P < 0.05$ ). There were no significant differences between the other isolates.

**Molecular weight range** The minimum molecular weight of the proteins recognised by canine IgG in all six isolates was 20 kDa. The maximum molecular weight varied between the isolates; it was 179 kDa for isolate 2, 185 kDa for isolate 1, 187 kDa for isolate 6, 190 kDa for isolate 5, 191 kDa for isolate 4 and 198 kDa for isolate 3. The median molecular weight was 43 kDa for isolates 1 and 5, 44 kDa for isolates 3, 4 and 6 and 45 kDa for isolate 2.

As the 75 percentile was not above 75 kDa in any of the six isolates, it was clear that most staphylococcal proteins recognised by canine IgG had a relatively low molecular weight. There were no significant differences between the six isolates.

**Intensity of bands** To assess the relevance of individual bands, the five most intense bands from each blot were selected for further analysis. Fig 3 shows the molecular weight distribution of the five most intense bands for each isolate of *S. intermedius*.

The band with the highest intensity in all six isolates was most often a protein with a molecular weight of approxi-

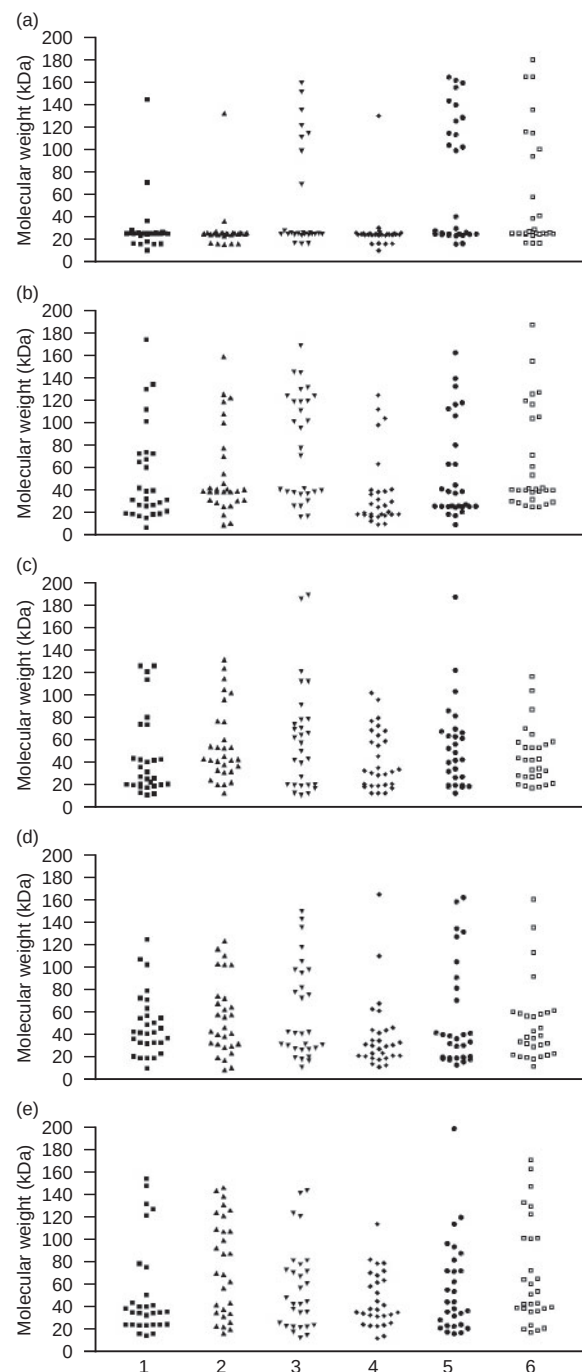


**FIG 3:** Comparisons of the molecular weights of the five most intense bands as assessed by digital image analysis; (a) the most intense band on each blot from each isolate; (b) to (e) the next four bands ranked according to intensity. Each data point represents a band from an individual dog. Numbers on the x axis indicate the six isolates of *Staphylococcus intermedius*

mately 28 to 29 kDa (Fig 3a). The next four bands ranked according to intensity showed a wider variation in molecular weight distribution, but there were clusters in the 20 to 60 kDa range (Fig 3b to e). In all six isolates the most intense IgG response was therefore to proteins with molecular weights less than 80 kDa.

There were significant differences in the range of molecular weights of the most intense band between isolates 1, 2 and 4 and isolate 5 ( $P < 0.001$ ), presumably owing to the presence of a cluster of higher molecular weight bands above 100

kDa in isolate 5 (Fig 3a). With the second most intense band, there were significant differences between isolates 3 and 4 ( $P<0.01$ ), but there were no other significant differences between the isolates.

## DISCUSSION

The IgG responses of these 21 dogs to staphylococcal proteins extracted from six different isolates of *S. intermedius*, as revealed by the protein banding patterns on SDS-PAGE gels stained with Coomassie blue, were very similar for all six isolates. Previous studies with cell extracts of different isolates of *S. aureus* also observed little difference in the SDS-PAGE patterns of separated proteins after Coomassie blue staining (Krikler and others 1986). In the present study, between 29 and 33 distinct bands were identified on SDS-PAGE gels of the *S. intermedius* extract, but these bands may not have been due to individual proteins. Crude extracts from whole organisms contain a large and complex mixture of proteins and an individual band may be due to a number of proteins with the same molecular weight. The numbers of bands on the immunoblots must therefore be interpreted with caution, because the IgG response represented by a particular band may be the response to a number of different proteins. The numbers of bands therefore indicate the minimum number of *S. intermedius* antigens that were recognised by canine IgG. The bands recognised by canine IgG with the greatest intensity were not the same proteins that were most prominent on the Coomassie blue-stained gels of the staphylococcal extracts. This is in accordance with findings in *S. aureus* and sera from human individuals (Bell and others 1987) and indicates that the most plentiful proteins in *S. intermedius* are not the most antigenic.

All the dogs showed an IgG response to multiple proteins from the six isolates examined, regardless of their state of health. Between 12 and 24 bands were observed, with a mean of approximately 17. The only difference detected was the presence of significantly more bands with isolate 2 than with isolate 6; the biological relevance of this difference is not known. It may merely indicate biological variation between the isolates but it could also indicate the production of additional antigens by isolate 2 that were not produced by isolate 6. Shearer and Day (1997) also observed IgG responses to staphylococcal antigens from a single isolate of *S. intermedius* in both healthy dogs and dogs with various skin diseases. Similar studies with sera from healthy people and protein extracts from *S. aureus* revealed reactions to many antigens (Bell and others 1987). Royan and others (2000) examined the ability of human sera to recognise macromolecules secreted by *S. aureus* and also found that a large number of proteins were recognised by IgG. Taken together, these results indicate that even in healthy dogs and people with no signs of skin disease, staphylococcal organisms produce a number of antigens that induce an antibody response.

The IgG response to all six isolates was predominantly targeted at proteins with molecular weights of less than 75 kDa, although antigens with molecular weights over 100 kDa were also recognised, especially in isolates 3, 5 and 6. Furthermore, the most intense bands which were considered to indicate the highest IgG concentrations, were also in the low molecular weight range. The most intense band in all six isolates typically had a molecular weight of 28 to 29 kDa. In human beings, IgG recognised proteins from *S. aureus* extracts with molecular weights of 84, 74, 64, 34, 31, 30, 28 and 18 kDa (Bell and others 1987). However, a study of patients infected with *S. aureus* and healthy people as controls showed that sera from most individuals contained IgG recognising a secreted protein of between 28 and 29 kDa (Royan and others 2000). The identity of these proteins is not known.

A staphylococcal protein that has received particular attention is protein A; it has a molecular weight of 42 kDa and is able to bind non-specifically to the Fc fragment of IgG of several species, including dogs (Langone 1982, Peng and others 1991, Kabir 2002). Some studies have suggested that most isolates of *S. intermedius* produce protein A (Cox and others 1986, Fehrer and others 1988) whereas others have found the opposite (Lachica and others 1979). In the present study, the protein A status of the six isolates was not known. However, in immunoblots, the presence of protein A would lead to a strong band with a molecular weight of 42 kDa. Although the serum of some of the dogs recognised a band at this molecular weight, it was not the strongest band in the majority of them.

The dogs' IgG responses to staphylococcal proteins of various molecular weights were similar to the responses observed by Shearer and Day (1997) in dogs with various skin diseases, which recognised proteins with molecular weights from 25 to 120 kDa, the 25 kDa protein being the most commonly recognised. However, in this study, such responses were observed not only in dogs with skin diseases but also in healthy dogs. The reason for this difference is not known, but it is probably associated with the different methods used. The fact that the control experiments showed that the IgG binding was specific indicates that it cannot be explained by non-specific binding. It is possible that the immunoblotting method used in this study was more sensitive because it recognised up to 24 bands on the immunoblots, whereas only nine bands were reported by Shearer and Day (1997).

In previous studies, the IgG response to staphylococcal extracts measured by ELISA was greater in dogs suffering from pyoderma than in healthy dogs (Morales and others 1994, Shearer and Day 1997; R. E. W. Halliwell, personal communication). In this study, the digital image analysis was only validated for intrablot analysis, that is, to compare the isolates, rather than interblot analysis, that is, to compare the dogs. However, it was clear that multiple bands were present in all the dogs, regardless of whether or not they had pyoderma. Further studies are therefore warranted to compare the IgG responses of different disease groups to determine whether the response to any of the staphylococcal antigens identified above is different in dogs with pyoderma than in the other groups. A direct comparison between the results of the semi-quantitative immunoblot system described here and the results of ELISAs would also provide useful quantitative data.

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