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COUNTER IMMUNO ELECTROPHORESIS AS RAPID AND QUANTITATIVE TEST IN DETECTION OF BETA AND EPSILON ANTITOXIN OF *CL. PERFRINGENS* TYPES B AND D IN COMPARISON WITH ELISA AND MOUSE PROTECTION TEST .

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SUMMARY

Counter immunoelectrophoresis (CIEP) assay was used as quantitative method for detection of Epsilon and Beta antitoxins in sera of vaccinated rabbits as compared with indirect enzyme linked immunosorbent assay (ELISA)in vitro and in vivo mouse protection test (SNT). For this reason eleven batches of clostridial vaccines were injected in rabbits. Results revealed, good correlation between SNT, ELISA and CIEP statistically. Therefore CIEP offers an alternative assay to in vitro ELISA and in vivo mouse protection test (SNT). CIEP is specific quicker, easier, economic as it takes 30 min instead of 3 hrs in case of ELISA. Also it needs small amount of serum and avoids the use of laboratory animals.

INTRODUCTION

Enterotoxaemia caused by Clostridium Perfringens types B and D is a fatal disease in sheep, goats , calves and occasionally, other species, (Blood et al., 1983). The major pathological agents are beta and Epsilon toxins of type B and D respectively (Uzal and Kelly, 1996). Therefore economic losses may be prevented successfully by adequate immuno prophylaxis (Rolle and Mayr, 1984) by vaccination (Uzal et al, 1996).

The potency of corresponding vaccines is currently tested by quantifying the antitoxins in sera of vaccinated rabbits (British Pharmacopoeia, 1996). The British pharmacopoeia prescribes a toxin neutralization assay using mice for the detection of antitoxin levels.

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Various authorities expressed the need for a reduction in the use of animals for experimental purposes (Council of Europe 1985). While there is no alternative to immunizing test animals when assessing the potency of vaccines, the protectivity can be evaluated in many cases by quantifying the antibody response using in vitro methods. Therefore various in vitro procedures have been used for the quantification of antitoxins of clostridial vaccines ; cell cultures have been used as indicators of excess toxicity in neutralization tests for several clostridial vaccines (Payne et al., 1994). However , cell cultures may not be sufficiently specific and may only indicate cytopathic entities. Enzyme linked immunosorbent assays have been developed for quantification of clostridial antitoxins in sera (Sojka et al., 1989, Wood, 1991), it was found to be quick, easy to perform and avoide the use of large numbers of animals as indicators for lethal and sublethal intoxication.

Counter immunoelectrophoresis (CIEP) has been used as a specific, rapid, sensitive, and easy accurate mean of inducing a precipitin reaction between a particular antigen and its antibody for the routine identification of various kindes of antigens (Edwards , 1971Cho and Langford, 1974; Hill et al., 1975 and Meher-Homji; 1975). Also, Naik and Duncan (1977) and Hendreson (1984) reported the use of counter immunoelectrophoresis for the detection of C. Perfringens toxins . Based on these reports this paper is aimed to describe (CIEP) for quantification of Epsilon and Beta antitoxins and compare the results with those obtained by SNT and ELISA .

MATERIALS AND METHODS

Vaccines :

Elven local and imported Batches of clostridial vaccines were used (5 batches of ultrabac vaccine, batch no. 12937220, 181945190, 179762390, 173716380 and 129372760; 3 batches of covexine 8 batch no., 301A, 411A and 436B, two batches of the polyvalent and one batch of the ivalent vaccine). Ultrabac vaccine consists of antigens of (C. chauvoei C. septicum; C. haemolyticum ; C.novyi type B; C.sordelli C. perf. types C. and D) produced by Pfizer Animal Health Co. USA. Covexine 8 consists of antigens of (C. septicam C.chauvoei, C. tetani, C. perf. types B & D, and C. novyi type D and B) produced by Schering - Plough Animal Health Co. New Zealand. the polyvalent vaccine consists of antigens of (C. septicam, C. chauvoei , C.perf. types B and D , C. novyi type B and C. tetani). The bivalent vaccine consists of antigens of (C. perf. types B & D). The last two vaccines are produced by Vet. Sera and Vaccines Research Institute , Abbassia , Cairo .

Animals :

2-1- lab animals :-

2-1-1- Rabbits :-

One hundred and ten white Boskat rabbits weighing (2.5 -3 kgm) were used and they

were divided into eleven groups each group consists of 10 rabbits. The animals in each group were injected with 2 doses of one of the eleven vaccines; 21 days apart as recommended by the manufacturer. Fifteen days post second injection, serum samples were collected separately from all groups. Polled sera from each group for each kind of vaccine were prepared and stored at -20°C till used.

2-1-2- white mice :

Five hundred white mice weighing about 15 - 20 gm were used for titration of antitoxins in sera of vaccinated rabbits and determination of the test dose of toxine used in toxin-antitoxin neutralization test.

3) toxins :

Standard toxins of CL-Perfringens type B and D (Beta and Epsilon toxins respectively) were obtained from Cent. Vet. Lab., New How , Weybridge, Surrey, U.K. The identity of the toxins was confirmed by toxinñ antitoxin neutralization test in mice carried out according to the method adopted by Sterne and Batty (1975). The test dose was considered as the lowest dilution of toxin neutralized by one international unit (IU/ml) of standard antitoxin.

4) Antitoxins

Standard Beta and Epsilon antitoxins were obtained from Cent. Vet. Lab., New How, Weybridge , Surrey, U.K. Each of the above contains 33750 IU/ml and 44.8 IU/ml respectively .

5) Preparation of standard rabbit serum :

Standard antisera against CL. perfringens type B and D were prepared according to the method adopted by Wood (1991) and standardized by toxin-antitoxin neutralization assay according to Sterne and Batty (1975). It contains 5 IU/ml and 10 IU/ml for Epsilon and Beta antitoxins respectively and it was used in ELISA assay .

6) Antitoxin assay by serum neutralization test in mice :

Samples of vaccinated rabbit sera were titrated using reference toxins and antitoxins as described in the British Pharmacopoeia (1996).

7) Enzyme linked Immunosorbent assay (ELISA):

The procedure was carried out according to Wood (1991) using antirabbit IgG, horseradish peroxidase and standard rabbit serum. The optical density was measured using microelisa reader at 492 nm.

ELISA TITER :

The reference serum method (RS) according to Williams (1987) for the transformation of absorbance value into a single figure representing the antibody titer was used depends on the use of reference serum with a known predetermined end-titer to calculate the titers of test sample on a proportional basis. This was ultimately established as

5 IU/ml & 10 IU/ml for Epsilon and Beta antitoxins respectively. Using this serum as standard reference, end titers of all samples in a specific test were calculated according to the following formula :

{ (OD of tested sample - OD of negative control) / (OD of reference serum - OD of negative control) } x titer of reference serum .

Counter Immunoelectrophoresis: (CIEP).

The procedure used for the CIEP was similar to that of Herbert (1970) and Henderson (1984). A plastic slide 8.5 x 14 cm was used to support the agar gel. The slide was layered with 20 ml of 0.7% agarose in 0.025 ionic - strength barbital acetate buffer (pH 8.6). Parallel rows of wells, 3mm in diameter, were cut 5mm apart in the agar. A slide of this size accommodated as many as three double rows with nine paired wells in each row , thus 27 samples could be analyzed for the presence of antitoxins in one slide. Standard antitoxins and antitoxins prepared in rabbits were titrated against standard toxins to determine the test dose of toxin (antigen) to be used in detection of antitoxins of unknown serum samples. 10ul of tested toxins diluted in barbital buffer pH 8.7 were placed in the rows of wells near the cathode. 10ul double fold dilutions of vaccinated animals serum samples diluted in barbital acetate buffer pH 8.7 were placed in wells near the anode . The elctrode vessel contained barbital acetate buffer of the same ionic strength, 0.025. Electrophoresis was carried out in an electrophoresis chamber at room temp. with constant current of 250 volts for a period up to 40 min using standard immuno electrophoresis unit, filter paper strips were used as an electrode wick. After electrophoresis the slide was removed and examined for the presence of precipitin bands between the two wells. If the precipitin band is not clear, the slide was removed to a humidity chamber and allowed to develop for 5 to 10 min. This process made the line very sharp and easily observable. Even a very faint precipitin line, not observable with the naked eye, could be intensified by overnight incubation in humidity chamber followed by staining with 0.1% commasi blue as described by Hirschifeld (1961). For permanent record, slides could be fixed as described by Uriel and Scheidegger (1955). A linear relationship existed between the concentration of antitoxins and the CIEP titer of antitoxin as determined by the highest dilution of antitoxin exhibiting a visible precipitin band. This allowed quantitation of antitoxins in unknown samples based on the titer obtained with a standard antitoxins of known concentration .

9) Statistical analysis :

It was done by T. test Microsoft and F. test Microsoft .

RESULTS AND DISCUSSION

Potency of clostridium perfringens type D and B vaccines must be capable of inducing (3.5- 5 IU/

ml) and 10IU/ml of neutralizing antibodies for Epsilon and Beta antitoxins respectively (Code Fedral Regulation, 1989 and British Phormacopoeia 1993). The serum toxin neutralization test in mice is still the statutory method for measuring the immunological response. Whilst this test is known to be sensitive, it is time consuming (2 : 3 days), expensive and requires the use of large number of mice. Also it can be rather imprecise as many variable factors like the condition of test animals and precision of inoculation technique, affect the results. Also, results are often presented as a range or require repetation, because the assay, necessarily, is performed using defined dilution, of test sera which either protect or not .

An alternative in vitro assays method needs to comply with two major qualifying factors :- detection of protective antitoxin only ; and precise quantification .

In accordance with the data previously mentioned, results obtained in Table (1) and Fig (1) where results between serum toxin neutralization assay and ELISA were compared showed that The mean antitoxin titer of E-antitoxin was 5.727 IU/ml with a range of 4 - 9IU/ml as measured by SNT while the mean antitoxin titer was 7.119 IU/ml with a range of 5.34 - 10.34 IU/ml as measured by ELISA. The mean antitoxin titer for beta antitoxin was 11.77 IU/ml with a range of 8 - 15 IU/ml measured by SNT and it was 12.82 IU/ml with a range of 8.75 - 16.7 IU/ml) measured by ELISA (Table 2) , Fig 2. From the above mentioned results, statistical analysis between mean antitoxin titer of Epsilon and Beta by SNT and

Table (1): Comparison between E. antitoxin titer measured by SNT, ELISA and CIEP in sera of vaccinated rabbits.

No.	Batch	Tests		
		SNT (IU/ml)	ELISA (IU/ml)	CIEP (IU/ml)
1	Local polyvalent	5	6.76	6
2	Ultrabac 12937220	4	5.34	5
3	Ultrabac 181945190	4	5.34	5
4	Ultrabac 179762390	4	5.37	5
5	Ultrabac 1733716380	4	5.36	5
6	Ultrabac 129372260	4	5.38	5
7	Local polyvalent	5	6.75	6
8	Covaxine 301 A	9	10.33	10
9	Covaxin 411 A	9	10.30	10
10	Covaxin 436 B	9	10.34	10
11	Local Bivalent	6	7.02	7
	Mean:	5.727	7.119	6.727

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Table (2): Comparison between B. antitoxin titer measured by SNT, ELISA and CIEP in sera of vaccinated rabbits.

No.	Batch	Tests		
		SNT (IU/ml)	ELISA (IU/ml)	CIEP (IU/ml)
1	Local polyvalent	15.0	16.7	16.0
2	Ultrabac 12937220	14.0	14.98	15.0
3	Ultrabac 181945190	14.0	15.43	15.0
4	Ultrabac 179762390	13.5	14.4	14.0
5	Ultrabac 1733716380	12.5	13.4	13.0
6	Ultrabac 129372260	14.0	15.01	15.0
7	Local polyvalent	8.0	8.85	9.0
8	Covaxine 301 A	10.0	11.07	11.0
9	Covaxin 411 A	10.0	11.1	11.0
10	Covaxin 436 B	10.0	11.3	11.0
11	Local Bivalent	8.0	8.75	9.0
	Mean:	11.77	12.82	12.64

ELISA test revealed good correlation where R2 was 0.991 and 0.992 respectively with no significant differences between the two tests. The present results agreed with Sojka et al (1989) and Wood (1991) , who found good correlation between ELISA and SNT (R = 0.93%) while in our study it was 0.99%. The reason for this difference may be due to the fact that in our test we used a purified activated Epsilon toxin instead of crude Epsilon toxoid as antigen (Sojka et al., 1989) or purified protoxin (Wood; 1991) which may have increased both the specificity and sensitivity. Our previous results revealed that ELISA is more economic by avoiding the use of large numbers of animals as indicator for lethal and sublethal intoxication, simple performance and takes less time than SNT.

Concerning the use of counter immunoelectrophoresis as an assay for quantitating antitoxin, the precipitin bands were detected after 20 - 30 min. It was noted that during the additional period of electrophoresis the precipitin bands observed after 20 min moved towards and finaly beyond the anodal well. It was concluded that the bands observed after 20 min were artefactual. These results differ from that of Henderson (1984) who found precipitin bands after 10 - 15 min between the intestinal contents and horse antitoxin and a rather longer time between the purified Epsilon toxin and horse antitoxin. This difference may be related to the variation in the electrophoresis conditions.

From the results obtained in tables (1) & (2), the mean antitoxin titer was 5.727 IU/ml (range 4 - 9 IU/ml) by using SNT and 11.77 IU/ml (range 8 - 15 IU/ml) for E- and B antitoxins respectively while, the mean antitoxin titer was 6.727 IU/ml

Fig.(1) Relation between E-antitoxin titer by SNT & ELISA

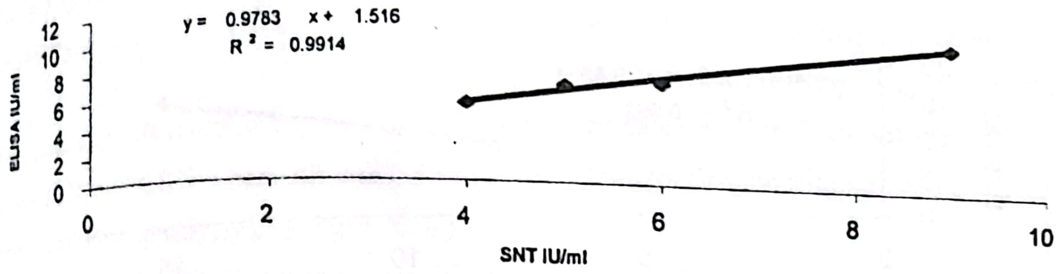


Fig. (2) Relation between E-antitoxin titer by ELISA & CIEP

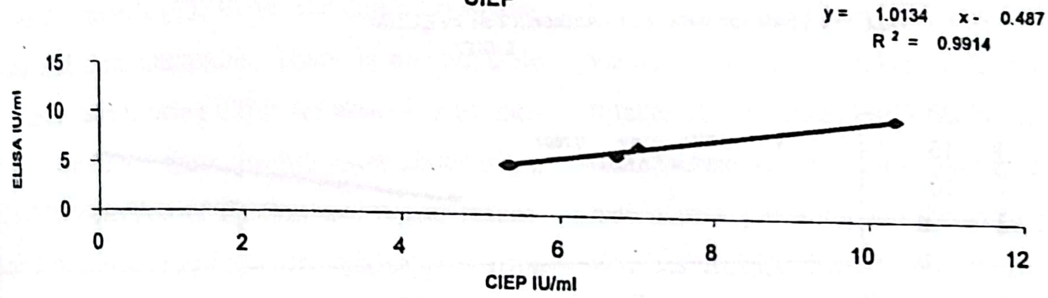


Fig.(3) Relation between E-antitoxin titer by SNT &CIEP

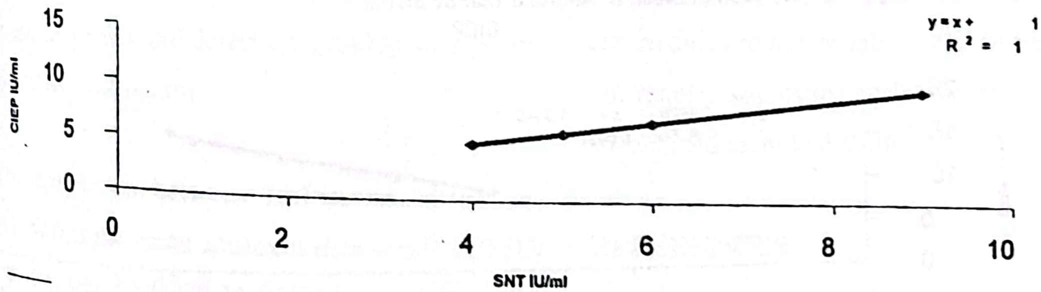


Fig.(4) Comparison of E-antitoxin titer by SNT,ELISA & CIEP

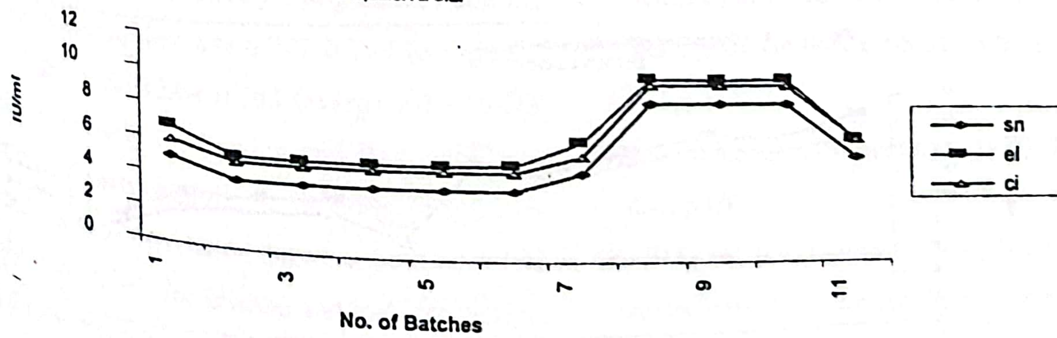


Fig.(5) Relation between B-Antitoxin titer by SNT &ELISA

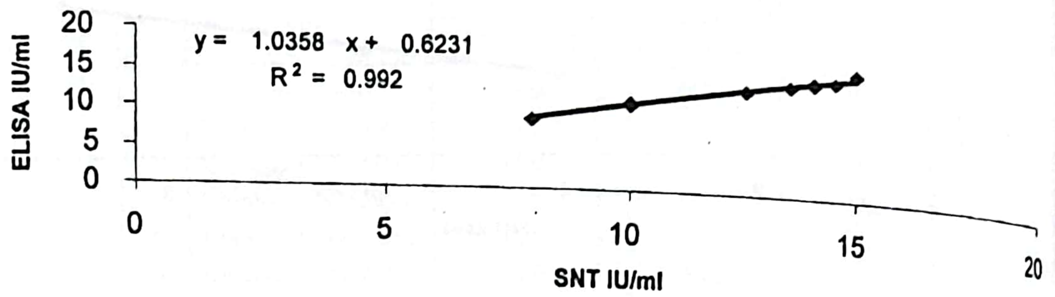


Fig.(6) Relation between B- Antitoxin titer by ELISA & CIEP

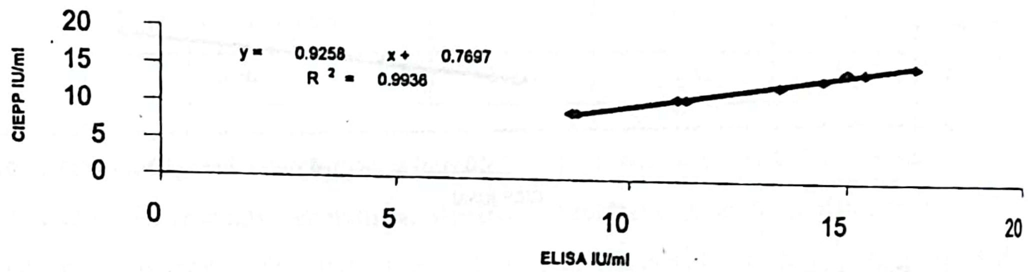


Fig.(7) Relation between B- Antitoxin titer by SNT & CIEP

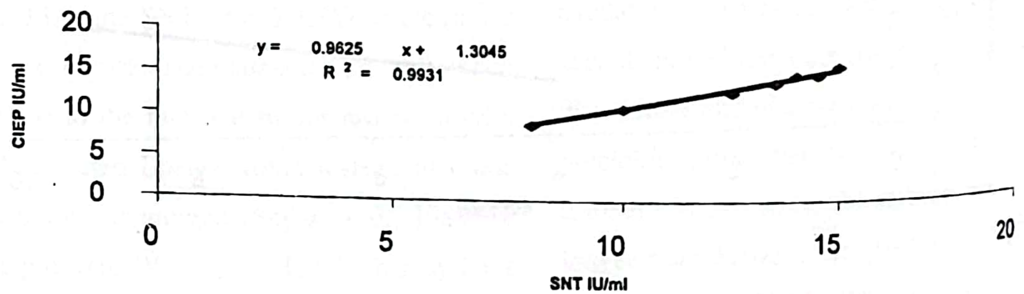
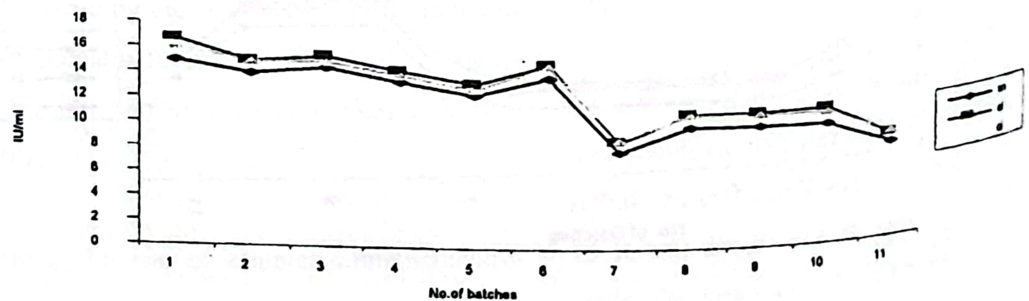


Fig.(8) Comparison of B-antitoxin titer by SNT,ELISA & CIEP



(rang 5 - 10 IU/ml) and 12.64 IU/ml (rang 9 - 16 IU/ml) by using CIEP for E- and B antitoxins respectively.

Statistical analysis revealed a good correlation between SNT and CIEP where R2 was (1 and 0.99 for E. and B respectively), (Fig 3 & 7) which means a high agreement between the results of the mouse protection assay (SNT) and counter immunoelectrophoresis (CIEP) for the detection of Epsilon and Beta antitoxins. There is no available literature about using CIEP for detection of antitoxins. However, there are literature about using CIEP for detection of Epsilon toxins and enterotoxins type A comparing with mouse protection assay. Naik and Duncan (1977), Henderson (1984) and Hornitzky (1989), found that CIEP may be used as an alternative to (SNT) as an aid in the diagnosis and detection quantity of Epsilon toxins and entrotoxin.

The correlation between ELISA and CIEP revealed that the mean antitoxin titer was 7.119 IU/ml (avarge 5.34 - 10.34 IU/ml) and 12.82 IU/ml (avarge 8.75 - 16.7 IU/ml) for Epsilon and Beta antitoxins respectively using ELISA and the mean antitoxin titer was 6.727 IU/ml (avarge 5 - 10 IU/ml) and 12.64 IU/ml (avarge 9.0 - 16 IU/ml) respectively for Epsilon and Beta antitoxins Statistical analysis of the results (Fig. 2 & 6) revealed a good cerrelation between both tests (R2 0.991 and 0.993, for Epsilon and Beta respectively) and insignificant differences between the two assays. Statistical analysis between the mouse protection test (SNT) , enzyme linked immunosorbent assay (ELISA) and counter immuno electrophoresis (CIEP) by using F-test Fig. (4 & 8) revealed no significant differences between the 3 tests for Epsilon antitoxin and B. antitoxin .

In conclusion, the use of CIEP offers an in vitro alternative to ELISA and in vivo mouse protection assay (SNT) where CIEP has a number of advantages. First it is quicker, easier and specific as it takes 30 min instead of 3 hrs in case of ELISA test . Second, CIEP quantitation based on determination of a precipitin line between paired wells whereas ELISA depends on intensity of colour and in SNT there is a slight gaping between the different dilution of serum. Third the CIEP is more economic as it needs small amount of serum, avoids the use of laboratory animals and does not require sophisticated laboratory equipment or specialized technical skills.

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